Biological and Chemical Detection of Trichothecene Mycotoxins of *Fusarium* Species

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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. citreoviride* Bougrje (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); diacetylvalenol 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 diacetylvalenol.

Among 20 mycotoxins tested, only the trichothecene compounds inhibited the uptake of radioactive amino acid in the reticulocyte bioassay 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. Trichothecene mycotoxins of Fusaria.**

| Type A                                                                 |
|-----------------------------------------------------------------------|
| ![Type A structure]                                                     |
| **Diacetoxyscirpenol**                                               |
| R<sub>1</sub>: OH  R<sub>2</sub>: OAc  R<sub>3</sub>: OAc  R<sub>4</sub>: H  |
| **T-2 toxin**                                                        |
| R<sub>1</sub>: OH  R<sub>2</sub>: OAc  R<sub>3</sub>: OAc  R<sub>4</sub>: COCH$_2$CH(CH$_3$)$_2$ |
| **Neosolaniol**                                                      |
| R<sub>1</sub>: OH  R<sub>2</sub>: OAc  R<sub>3</sub>: OAc  R<sub>4</sub>: OH  |
| **HT-2 toxin**                                                       |
| R<sub>1</sub>: OH  R<sub>2</sub>: OAc  R<sub>3</sub>: OAc  R<sub>4</sub>: COCH$_2$CH(CH$_3$)$_2$ |

| Type B                                                                 |
|-----------------------------------------------------------------------|
| ![Type B structure]                                                     |
| **Nivalenol**                                                         |
| R<sub>1</sub>: OH  R<sub>2</sub>: OH  R<sub>3</sub>: OH  R<sub>4</sub>: OH  |
| **Fusarenon-X**                                                      |
| R<sub>1</sub>: OH  R<sub>2</sub>: OAc  R<sub>3</sub>: OH  R<sub>4</sub>: OH  |
| **Diacetylnivalenol**                                                 |
| R<sub>1</sub>: OH  R<sub>2</sub>: OAc  R<sub>3</sub>: OAc  R<sub>4</sub>: OH  |

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**NOTES**

**Type A**

**Diacetoxyscirpenol**

**T-2 toxin**

**Neosolaniol**

**HT-2 toxin**

**Type B**

**Nivalenol**

**Fusarenon-X**

**Diacetylnivalenol**
standardsolution
ethylacetate-n-hexane
toxins
observed
vent
ultraviolet
calandchemical
A-type
(90:5:5).
Type
Type
VOL.
TABLE1.
T-2toxin
Diacetoxyscirpenol
Diacetylnivalenol
Fusarenon-X
HT-2 toxin
Mycotoxins
Withthe
a
Abbreviations:
limit
25,1973
B
B
Trichothecenesuptake
with
light
and
required
HSO4
microlitersofthe
membranes
for
20%
heating
and
then
heating
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-iso-propanol-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
Rf
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
conducted
a
careful
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
14C-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.
Nivae,
F.
epispheeria,
etc.).
(iii)
The
former
fungi
were
distributed
in
the
northern
districts
of
Japan
and
the
latter
fungi
were
in
the
southwestern
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.
solanum
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
poisonant,
12-13
epoxy
trichothecenes
of
Fusarium
species.

**Table 1. Comparative toxicity of trichothecenes to rabbit reticulocytes and mice**

| Trichothecenes     | ID_{50}^{*} to {^{14}C}-leucine uptake in reticulocytes (µg/ml) | LD_{50}^{*} to male mice of ddY, 6-week-old (mg/kg) |
|--------------------|---------------------------------------------------------------|--------------------------------------------------|
| Type A             |                                                               |                                                  |
| T-2 toxin          | 0.03                                                          | 5.2                                              |
| Diacetoxyscirpenol | 0.03                                                          | 22.0                                             |
| Neosolaniol        | 0.30                                                          | 14.5                                             |
| HT-2 toxin         | 0.03                                                          | 9.0                                              |
| Type B             |                                                               |                                                  |
| Nivalenol          | 3.0                                                           | 4.1                                              |
| Fusarenon-X        | 0.30                                                          | 3.3                                              |
| Diacetylneosolaniol| 0.10                                                          | 9.6                                              |

* 50% inhibitory dose.
* 50% lethal dose, given intraperitoneally.


**Table 2. Thin-layer chromatography of trichothecenes**

| Mycotoxins      |          |          |          |          | Color after spray |
|-----------------|----------|----------|----------|----------|-------------------|
|                 | CM       | EH       | BA       | CPE      | CEE               | Visible light | UV light      |
| Type A          |          |          |          |          |                   |                |               |
| 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          |          |          |          |          |                   |                |               |
| 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 |
| Diacetylneosolaniol | 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-iso-propanol-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, HT-2 toxin, diacetoxyscirpenol |
|                 | M-13-2     | +        | Neosolaniol, T-2 toxin, diacetoxyscirpenol  |
|                 | M          | -        |                                            |
|                 | No. 1 (clover) | +  |          |
|                 | No. 2 (alfalfa) | -  |          |
| F. rigidiusculum | M-1-3     | ++       | Neosolaniol, T-2 toxin, diacetoxyscirpenol  |
|                 | M-10-1     | -        |                                            |
|                 | M-13-3     | -        |                                            |
|                 | M          | +        |                                            |
| F. roseum       | M-2-5      | +        | Diacetoxyscirpenol                          |
| "culmorum"      | M-3-2      | +        |                                            |
| "avenaceum"     | M-7-1      | +        |                                            |
| "scirp"         | M-7-2      | -        |                                            |
| "avenaceum"     | M-8-1      | ++       | Neosolaniol, T-2 toxin, diacetoxyscirpenol  |
| "culmorum"      | M-11-1     | ++       | Neosolaniol, T-2 toxin, diacetoxyscirpenol  |
|                 | M-13-1     | -        |                                            |
|                 | M-14-2     | +        | diacetoxyscirpenol                          |
|                 | M-15-2     | -        |                                            |
|                 | 70-K-11    | ++       | Neosolaniol, T-2 toxin, HT-2 toxin, diacetoxyscirpenol |
|                 | M          | -        |                                            |
|                 | Abashiri-1 | +        | Neosolaniol, T-2 toxin                      |
|                 | Abashiri-5 | -        | Neosolaniol, T-2 toxin                      |
|                 | Asahikawa-1| +        | Neosolaniol, T-2 toxin                      |
|                 | Asahikawa-4| ++       | Neosolaniol, T-2 toxin                      |
|                 | Asahikawa-6| ++       | Neosolaniol, T-2 toxin                      |
|                 | Asahikawa-7| -        | Neosolaniol, T-2 toxin                      |
|                 | R 2029     | +        | Neosolaniol, T-2 toxin                      |

Continued on next page
TABLE 3—Continued

| Fungus              | Strain          | Bioassay | Trichothecces on thin-layer chromatography |
|---------------------|-----------------|----------|------------------------------------------|
|                     |                 | Reticulocyte* | Mice*                      |                                    |
| **F. gibbosum**     | M-14-1          | –         | –                                        |                                      |
|                     | Abashiri-6      | +         | –                                        | Fusarenon-X, nivalenol             |
|                     | Asahikawa-2     | –         | +                                        |                                      |
| **G. zeae**         | Ohsita-II       | +         | +                                        |                                      |
|                     | Ishii           | –         | +                                        |                                      |
|                     | NRRL 2830       | –         | –                                        |                                      |
|                     |                 | –         | –                                        |                                      |
| **F. tricinctum**   | M               | –         | –                                        |                                      |
|                     | NRRL 3249       | –         | –                                        |                                      |
|                     | NRRL 3299       | +++       | +++                                      | Neosolaniol, T-2 toxin, HT-2 toxin, diacetoxyscirpenol |
|                     | Abashiri-2      | ++        | ++                                      | Neosolaniol, T-2 toxin, HT-2 toxin, diacetoxyscirpenol |
|                     | R 2031          | ++        | ++                                      | Neosolaniol, T-2 toxin, HT-2 toxin, diacetoxyscirpenol |
|                     | R 2031          | ++        | ++                                      | Neosolaniol, T-2 toxin, HT-2 toxin, diacetoxyscirpenol |
| **F. sporotrichioides** | M-1-4          | ++        | ++                                      | Neosolaniol, T-2 toxin, diacetoxyscirpenol |
|                     | M-1-5           | ++        | ++                                      | Neosolaniol, T-2 toxin, diacetoxyscirpenol |
|                     | NRRL 3510       | +++       | +++                                      | Neosolaniol, T-2 toxin, diacetoxyscirpenol |
| **F. oxysporum**    | M-4-1           | –         | –                                        |                                      |
|                     | M-15-1          | +         | –                                        |                                      |
|                     | M               | +         | –                                        |                                      |
|                     | NRRL 1943       | –         | +                                        |                                      |
| “niveum”            | Melon-1         | ++        | ++                                      | Fusarenon-X, diacetylnivalenol      |
| “niveum”            | Melon-2         | ++        | ++                                      | Fusarenon-X, diacetylnivalenol      |
|                     | 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 14C-leucine was assayed in concentrations of 10 and 100 ng/ml.

a The lethal effect of each crude toxin to mice was assayed after intraperitoneal administration of 10 and 40 mg/10 g. The relative toxicity in both systems was cited.

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