Studies on the Population of Toxigenic Fungi in Foodstuffs

VI. Histopathologic Changes in Mice Caused by Toxic Metabolites of Fungi Isolated from Domestic Rice

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During a population survey of toxic fungi in foodstuffs, two strains of highly toxic fungi and nine strains of mildly toxic fungi were detected. Toxic metabolites of these 11 strains of fungi were administered subcutaneously to male mice weighing 15-18 g, respectively, and seven days after the single administration, experimental animals were killed and histopathological observations of various organs were carried out.

The nature and the degree of the induced histopathological changes of various organs of mice were thoroughly investigated and described. Hepatic injuries, e. g., irregularity of the nuclear size of hepatic cells, occasional mitotic figures, and inflammatory changes were commonly recognized as an induced pathologic change by all fungal toxic metabolites used in this study, however, the hepatic changes seemed to be nonspecific reactions. The most conspicuous hepatic injuries were noticed in mice administered toxic metabolites of the *Penicillium islandicum*. Nephrotoxicity of the *Penicillium islandicum*, and neurotoxicity of the *Penicillium decumbens* and the *Penicillium cyclopium* were indicated.

As it has been demonstrated in the previous toxicity study, it was noteworthy that even with toxic metabolites obtained from the same fungus, different methods of obtaining the toxic metabolites caused a different pathological effect. Individual differences in susceptibility among experimental animals to the fungal toxic metabolites were also observed.

Introduction

From the extensive reviews of the mycotoxin problem by Forgacs¹⁾²⁾, Borker et al.³⁾, Raymond⁴⁾ and Campbell⁵⁾, it is obvious that the need for mycotoxicological studies is increasing and the emphasis must be placed on human health rather than animal feed sanitation. Up to the present time, three mycotoxins (islanditoxin, aflatoxin and ochratoxin) have been disclosed as extremely potent hepatotoxigenic and hepatocarcinogenic agents⁶⁾.

Islanditoxin, one of the metabolites of *Penicillium islandicum*, was discovered in Japan by Tsunoda⁷⁾ and Kobayashi et al.⁸⁾ in 1950-1958, and it has been shown to cause proliferation of bile ducts and cirrhosis of the liver of rats and mice. The second, aflatoxin, was firstly found in Brazilian peanuts meal contaminated with *Aspergillus flavus* in Britain⁹⁾ in 1961. It has been incriminated as a causal agent for periportal necrosis with bile duct proliferation in the livers of ducklings and rats. Quite recently, ochratoxin A was obtained from *Aspergillus ochraceus* by Scott¹⁰⁾ and Van der Merwe et al.¹¹⁾ in South Africa. Theron et al.¹²⁾ described its pathological characteristics in the livers of ducklings, e. g., hyaline degeneration of the hepatic cells with focal necrosis, but without bile duct proliferation. Another mycotoxin, patulin, was investigated by Yamamoto¹³⁾ in a case of serious food poisoning among daily cattle fed with moldy

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malt feed infected with a toxigenic strain of *Penicillium urticae*. The post mortem examination disclosed the presence of hemorrhagic foci in the central nervous system. These pathological findings suggest that there are certain specific individual hepatic changes attributable the toxic effect of respective mycotoxins. Therefore, it must be emphasized that histopathological study of mycotoxins administrated animals may give us precise and detailed information concerning the nature of the toxicity of mycotoxins.

In a previous paper⁽⁴⁾, the authors reported the results of toxigenicity tests for various metabolites of fungi which had been isolated from domestic rice harvested in 1965. Of the total of 82 representative strains of fungi, two strains of highly toxic and eight strains of mildly toxic fungi were detected. The object of this paper is to report on the results of histopathological investigation of various organs of mice which were administrated the toxic fungal metabolites subcutaneously.

Materials and Methods

Ten strains of fungi isolated from milled rice by the authors, that had been determined to be toxic (Table 1), were used for this study. In addition, *Aspergillus ochraceus* Wilhelm (SF-13-1), isolated from a soybean flour and identified as a

mildly toxic to mice by the authors⁽⁵⁾, was also submitted for this survey.

Following the method for the determination of toxicity previously described, the methanol extracts of fungi and culture filtrates were prepared and administrated subcutaneously in a single dose to male mice weighing 15-18 g. Mice were killed and autopsies performed 7 days after the administration of fungal metabolites. Organs were fixed in 10% formalin solution. Paraffin sections were made and stained with hematoxylin and eosin, and periodic acid Schiff reaction.

Tissue reactions of the liver, the heart, the thymus, the lung, the kidney, the adrenal, the spleen, the pancreas, the sternal bone marrow, the forebrain, the cerebellum, the stomach and the intestine were investigated.

Control mice were killed seven days after injection with propylene glycol, peptone glucose medium and Czapek solution medium without fungal inoculation, respectively, and studied by the same procedure.

Results

Summary of histopathologic findings in various organs of the experimental animals are shown in Table 2.

Histopathologic findings of each experimental

Table 1. Materials used for the histopathological investigation following the toxigenicity test with metabolic substances of fungi isolated from domestic rice

Fungal species	Strain No. (MR-)	Liquid culture		Solid culture	
		Toxicity (Dose 0.5 ml/mouse)		Toxicity (Dose mg/kg)***	
		PG-medium*	CZ-medium**	250	1250
Penicillium:					
<i>P. cyclopium</i> West. var. <i>echinulatum</i> T. & R.	188-1	—	±	—	+
<i>P. decumbens</i> Thom	163-2	—	±	—	+
<i>P. expansum</i> Link	72-3	±	—	—	+
"	212-3	—	‡	+	
<i>P. islandicum</i> Sopp	202-6	‡	±	+	‡
<i>P. martensii</i> Biourge	131-1	±	—	—	+
<i>P. roqueforti</i> Thom	212-2	±	—	±	+
"	219-2	—	—	±	±
Aspergillus:					
<i>A. mangini</i> Thom & Raper	208-4	±	±	—	
<i>A. versicolor</i> (Vuil.) Tirab.	163-1	—	—	+	+

Symbols: — not effective

± reduction of body weight was noticed

+

‡ all animals tested died

* Peptone-glucose medium filtrate

** Czapeck solution medium filtrate

*** Methanol extracts

groups will be described briefly as follows.

1. Control mice.

Propylene glycol injected group: Even an irregularity of the nuclear size of the hepatic cells and occasional mitotic figures were recognized, no periportal cell infiltration nor intralobular granulomatous changes were disclosed in the liver. Congestion of the liver, the kidney, the adrenals, the pancreas, the heart, the thymus and of the lungs were the common findings in this group. The brain, the bone marrow, the spleen, the stomach and the intestine showed no unusual findings.

Peptone glucose medium injected group: Slight periportal cell infiltration, consisting chiefly of mononuclear cells, and granulomatous changes in the hepatic lobulus were revealed in some mice of this group. The latter were scattered throughout the hepatic lobulus, however, most of them were small in size and focal. It seemed that the essential feature of the granulomatous change was almost identical to the murine viral hepatitis. Histologic findings of the other organs were essentially as those observed in the propylene glycol injected mice.

Czapek solution medium injected mice: No unusual findings were observed in the organs investigated.

From the above findings in the control mice, it could be assumed that single subcutaneous administration of the propylene glycol, peptone glucose medium and Czapek solution medium provoked injurious effect in minor degree to the mice. However, no specific histologic changes were recognized in the control animals.

2. Mice injected with metabolites of *Penicillium cyclopium* West. var. *echinulatum* T. & R.

In the liver, periportal cell infiltration and granulomatous changes and irregular size of hepatic nuclei were more predominant than in the control animals. Bronchitis and pneumonia were commonly associated with this group. The most remarkable finding of this group was the spongy state of the cerebellar medulla, pontine and internal capsule of the forebrain (cf. Fig. 9), however, this findings were not revealed in the group of animals administered methanol extract at the rate of 1,250 mg per kilogram body weight. Several giant tubuli and mitotic figures of the tubular epithelium of the kidney were observed in the mice administered Czapek solution medium filtrate (cf. Fig. 8).

3. Mice injected with fungal metabolites of *Penicillium decumbens* Thom (MR-163-2)

The spongy state of the pons, cerebellar medulla and internal capsule of the forebrain was the most noticeable finding in this group (Fig. 9). No specific relation between the presence and/or the degree of

this spongy state and the hepatic pathology was revealed.

4. Mice injected with fungal metabolites of *Penicillium expansum* Link (MR-72-3 and MR-212-3)

The hepatic changes of the MR 72-3 group was more conspicuous than that of the MR-212-3 group. Bronchitis and pneumonia, however, were more frequent complications in the latter group than in the former. Giant tubulus and mitosis of the tubular epithelium of the kidney was observed in the groups administered peptone glucose and Czapek solution medium (Fig. 8), but was not observed in the groups injected with methanol extract.

5. Mice injected with fungal metabolites of *Penicillium islandicum* Sopp (MR-202-6)

A remarkable ballooning of hepatic cells of periportal and midzonal regions was observed in the liver (Fig. 5). The finding was characteristically observed only in the mice administered peptone glucose medium filtrate. Periportal cell infiltration and granulomatous changes in the hepatic lobuli were not conspicuous in this group. Dilatation of the distal convoluted tubulus of the kidney was also noticed and giant tubulus with peculiar tubular epithelium was observed in some animals of this group (Fig. 7).

6. Mice injected with metabolites of *Penicillium martensii* Biourge (MR-131-1)

Mitotic figures of tubular epithelium, especially in the proximal convoluted tubulus, of the kidney were frequent and characteristic finding in this group. Mitosis of Kupffer's cells was noticed in the granulomatous hepatic lesion (Fig. 3).

7. Mice injected with fungal metabolites of *Penicillium roqueforti* (MR-212-2 and MR-219-2)

Giant tubulus and marked dilatation of the convoluted tubulus of the kidney were observed frequently in this group (Fig. 6). Conspicuous irregularity of the nuclear size of the hepatic cells was remarked in this group (Fig. 2). Meningoencephalitis was disclosed in some mice of this group.

8. Mice injected with fungal metabolites of *Aspergillus mangini* Thom & Raper (MR-208-4)

Periportal inflammatory changes and granulomatous change in hepatic lobuli were frequent findings in this group. Submucosal mononuclear cell infiltration of the intestine was also commonly observed.

9. Mice injected with fungal metabolites of *Aspergillus versicolor* (Vuil.) Tiraboschi (MR-163-1)

A widespread submassive necrosis of the liver was noticed in one mouse which had been administered a methanol extract at the rate of 250 mg per kilogram body weight (Fig. 4). No the other mice of this group showed such a finding.

Table 2. Summary of Histopathological

Findings in Mice

<i>P. islandicum</i> MR-202-6	<i>P. martensii</i> MR-131-1	<i>P. roqueforti</i> MR-212-2	<i>P. roqueforti</i> MR-219-2	<i>A. mangini</i> MR-208-4	<i>A. vers.</i> MR-163-1	<i>A. ochr.</i> SF-13-1
M1 M2 PG CZ	M1 M2 PG CZ	M1 PG CZ	M1 M2 PG CZ	M1 M2 PG CZ	M1 PG CZ	C1 C2
3 4 4 3 1 3 1 2 1 1 2 1 3 1 3 4 3 1 1 1 1 1 4 1 3 4 4 3	5 3 5 5 2 2 1 1 1 1 1 3 1 1 3 2 3 2 1 1 3 2 1 5 1 5 4 1 3 5 3 5 4	5 4 5 2 2 1 1 1 1 4 2 1 1 1 1 1 2 1 4 2 1 1 2 4 1 1 4 4 5	4 5 5 4 2 2 3 1 1 2 3 1 1 1 1 1 1 2 3 2 1 1 2 3 3 1 2 2 4 4 3 3	5 5 5 5 2 2 3 2 1 2 2 3 4 3 2 1 2 4 2 1 2 4 3 4 2 1 2 1 3 1 1 5 4 5 4	4 4 5 1 1 2 1 1 1 2 1 1 1 1 2 1 2 3 1 2 1 4 2 1 2 2 4 4 5	5 5 1 1 2 1 2 1 3 4 2 5 3 5 5
2 4 4 3 1 2 1 1 1 1	3 2 5 5 2 5 4 1 1	4 4 4 1 1 4	2 4 4 3 1 1	4 4 5 4 2 2 2	5 5 4 1 1 1 2 1	5 5 5 5 1 1
3 4 4 3 3 4 4 3 1 4 1	5 3 5 5 5 3 5 4 1 2 4 2	5 5 5 5 5 5 1 2 3 2	4 5 4 3 4 5 4 3 1 1 2	5 5 5 5 5 5 5 5 1	5 5 5 5 5 5 1	5 5 5 5 1 1
2 4 4 3 2 4 4 3	5 3 5 5 5 3 5 5	4 5 4 4 5 4 1	4 5 4 4 4 5 4 4	4 5 5 4 2 1 2	5 4 5 5 4 5	5 5 5 5
3 4 4 3 2	5 3 5 5 1	5 5 5 2 1 2	4 5 5 4	5 5 5 5 5 4 3	5 5 4	5 5 4 5 2 2
3 4 4 3	5 3 5 5	3 4 4 2	4 5 5 3	3 5 5 4 1	5 5 5 3 1	5 5
3 3 3 3 3 3 3 2 1 1	5 3 5 5 5 3 5 5 1 2 1 3 2 2 2 1 1	5 5 5 5 5 5 1 1 1 1 2 2 2	4 5 5 4 4 3 5 4 1 1 4 2 4 4 2 1 1	4 5 5 5 4 5 5 5 1 2 2 2 1 2 1 1	5 5 5 5 5 5 1 1	5 5 5 5 1 2 1
3 4 4 3 1 1	5 3 5 5 1 2 2 2	5 2 5	4 4 5 4 1 1 1	5 5 5 3 1 1	5 5 5	5 5 5 5
3 4 4 3 1 1 2 3	4 3 5 3 2 2	3 5 5 2 1	4 4 4 3 1 1	5 5 5 3 1	5 5 4 1 2	4 1 1

1250 mg/kg; PG: Peptone glucose medium filtrate; CZ: Czapek solution filtrate

findings were disclosed, and the mice, in which corresponding findings were not disclosed, were

Irregular staining of the myocardial muscle was noticed in the mice administrated with the methanol extract.

10. Mice injected with metabolites of *Aspergillus ochraceus* Wilhelm (SF-13-1)

Mitotic figure of the hepatic cells was observed most abundantly in this group (Fig. 1). Conspicuous cell infiltration, which composed chiefly of neutrophils, was noticed in the submucosal layer of the intestine.

The thymus, the adrenal, and the sternal bone marrow showed no remarkable changes, and no tumor formation in any of the organs was disclosed in all mice investigated. Because no specific stains for neuropathological study performed, future study will be necessary for the evaluation of the findings of the nervous system.

Bronchitis, peribronchitis and bronchopneumonia were the common incidental findings among the mice used in this experiment. No specific organisms, e.g. mycoplasma, fungi or bacteria, were disclosed from those foci.

No specific pathological changes were revealed in the spleen, the pancreas and gastrointestinal tracts.

Discussion

It is interesting that the pathological effect of even fungal metabolite obtained from the same fungus was different when the metabolites were extracted by different methods. Individual differences in susceptibility to the mycotoxin were suggested in this experiment.

Even in the control mice, irregularity of the nuclear size of the hepatic cells and occasional mitotic figures were revealed. However, these changes were observed more predominantly in the experimental groups. It has been stated that several viral agents has been recovered from mice in association with hepatitis, and some of the viruses tend to produce latent infections which may be activated by experimental procedures¹⁶⁾. Therefore, the findings observed in the liver, e.g. slight periportal cell infiltration and focal granulomatous changes in the hepatic lobules, seemed to be an identical finding to the murine viral hepatitis. These findings were observed even in the control animals, however, it was more predominantly observed in the experimental groups, in general. It is conceivable that fungal metabolites used in this experiment would provoked hepatic injury in some extent, and among the present experiments it can be concluded that fungal metabolites from the *Penicillium islandicum* Sopp cultured in peptone glucose medium is the most hepatotoxic.

Bile duct proliferation has been documented as

the characteristic finding in aflatoxin administrated rats^{17),18)} and ducklings^{19,20)}. It was not recognized in the present study. No aflatoxin-like activity, therefore, was caused by the fungal metabolites used for this study. Vacuolization of the liver cells has been reported in toxic *Aspergillus clavatus* Desm²¹⁾. Marked vacuolization of the hepatic cells was revealed only in the mice administrated with fungal metabolites of *Penicillium islandicum* Sopp.

Peculiar tubular changes, e.g. marked tubular dilatation and peculiar epithelial morphology and arrangement, were observed in the mice administered peptone-glucose medium filtrate of the *Penicillium islandicum* Sopp. An identical finding has been reported in mice administered methanol extract of *Aspergillus oryzae* (Ahlb.) Cohn²²⁾. Tubular necrosis, which has been observed in rats administered ochratoxin A and sterigmatocystin²⁰⁾, was not recognized in the experimental mice investigated in the present study.

Spongy state of the cerebellar medulla, the pons, and of the internal capsule of the forebrain was the predominant and peculiar finding for the fungal metabolites of *Penicillium decumbens* Thom and *Penicillium cyclopium* West. Except for the mice administrated with *Penicillium roqueforti* Thom, no meningitis nor encephalitis were found among the experimental groups.

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Fig. 1. Marked irregularity in nuclear size and mitotic figure of the hepatic nuclei

No remarkable Kupffer's cell mobilisation

H. & E. $\times 400$

Liver of mouse No. 7618 administered a chloroform extract of *Aspergillus ochraceus* Wilhelm (SF-13-1) isolated from soybean flour at a rate of 250 mg per kilogram body weight

Fig. 2. Conspicuous irregularity in nuclear size of hepatic cells

H. & E. $\times 400$

Liver of mouse No. 7367 administered peptone glucose medium filtrate of *Penicillium roqueforti* Thom (MR-212-3)

Fig. 3. Granulomatous change in the hepatic lobulus

Mitosis of Kupffer's cells is demonstrated in the center

H. & E. $\times 400$

Liver of mouse No. 6444 administered Czapek solution medium filtrate of *Penicillium martensii* Biourge (MR-131-1)

Fig. 4. Massive necrosis of the hepatic parenchyma

H. & E. $\times 40$

Liver of mouse No. 6666 administered methanol extracts of *Aspergillus versicolor* (Vuil.) Tirab. (MR-163-1) at the rate of 250 mg per kilogram body weight

Fig. 5. Conspicuous vacuolar changes of the peripheral and midzonal regions of the hepatic lobulus

H. & E. $\times 40$

Liver of mouse No. 6279 administered peptone glucose medium filtrate of *Penicillium islandicum* Sopp (MR-202-6)

Fig. 6. Marked dilatation of the tubulus

H. & E. $\times 100$

Kidney of mouse No. 7519 administered Czapek solution medium filtrate of *Penicillium roqueforti* Thom (MR-219-2)

Fig. 7. Marked dilation of the renal tubulus and the epithelium showing peculiar morphology and arrangement

H. & E. $\times 100$

Kidney of mouse No. 6279 administered peptone glucose medium filtrate of *Penicillium islandicum* Sopp (MR-202-6)

Fig. 8. Giant tubulus with mitotic figure of the tubular epithelium

H. & E. $\times 100$

Kidney of mouse No. 7505 administered peptone glucose medium filtrate of *Penicillium expansum* Link (MR-212-3)

Fig. 9. Spongy state of the cerebellar medulla

H. & E. $\times 100$

Cerebellum of mouse No. 6490 administered peptone glucose medium filtrate of *Penicillium decumbens* Thom (MR-163-2)