STUDY OF TOXINS ISOLATED FROM GRAIN INFECTED WITH *FUSARIUM SPOROTRICHIOIDES*

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ABSTRACT

Experiments with mice showed that an aqueous extract of barley grain which had been attacked by *Fusarium sporotrichioides* is blastogenic.

There was a significant association between tumour-bearing and alpha-foetoprotein when a sensitive immunoautoradiographic method was used. The chromatographic examination confirmed that the R_f value and the colour of fluorescence of the extracts of grain infected with *Fusarium sporotrichioides* were substantially different from the aflatoxins used as controls or produced by *Aspergillus flavus*.

The discovery at the end of the 1930s, by a group of Soviet research workers headed by Drobotko[1], of the etiology of stachybotryotoxicosis was the beginning of a new stage in the understanding of the role played by mycotoxins in the etiology of a number of chronic non-communicable diseases.

The pathogenic effects of mycotoxins on human health became particularly important after Sarkisov, Kvasnina[2] and Rubinstein[3] established, in the post-war years, that the mould *Fusarium sporotrichioides* was the cause of a severe disease known at that time as 'septic sore throat'. After careful study of the clinical course of the disease and the establishment of its etiology it was renamed 'alimentary toxic aleukia' (ATA).

A further development in this type of research was the discovery by British workers of the etiology of 'turkey-X disease' and the discovery in animal experiments of the carcinogenic properties of metabolites of the mould *Aspergillus flavus* which became known as 'aflatoxins'. However, while there is no doubt that aflatoxins do have a carcinogenic effect on experimental animals, their significance in human pathology is still to be proved.

In connexion with the data on aflatoxins, the question naturally arose of the blastogenicity of products of the vital activities of moulds of another genus, *Fusarium*, which attack cereals when they are not properly stored. The metabolites undoubtedly play a part in human pathology.

Trials were carried out on mice to discover the blastogenic properties of

an aqueous extract of barley grain infected with *F. sporotrichioides* No. 63†. Ninety-seven P_1 hybrid mice (C57 × CBA), both male and female, were used in the experiment at the age of about 2½ months.

To prepare the extract, 150 ml of water were poured on to 25 g of autoclaved grain and the mixture was mechanically shaken for 3 hours. The solution was then filtered off. A preliminary experiment had shown that the maximum tolerated dose of this extract in chronic experiments on mice was 0.5 ml. At does of 1.0 and 0.8 ml the animals quickly died with symptoms of toxicosis.

In the main experiment the mice were each given 0.5 ml of the extract through a gastric sonde five times a week for a year. Altogether during the year the mice were given about 125 ml of the extract. Twelve mice died in the early stages and were not included when the results were summarized.

Six months after completion of the experiment (i.e. 18 months after it had begun) all the surviving animals were sacrificed and a microscopical examination was made of the liver and lungs and also of other macroscopically altered organs and tissues. The results of the experiment are given in *Table 1*.

As will be seen from the data in the table, the extract caused a statistically significant increase in lung adenomas in general, and particularly in the males. Lung adenomas occurred in 4 (11.4 per cent) of the 35 controls and in 18 (36.0 per cent) of the 50 experimental animals. In males the respective percentages were 5.8 and 36.8. Although a statistically significant increase in lung adenomas was not found in females they were nevertheless more frequent than in the controls (35.4 per cent as against 16.6 per cent) and the only adenocarcinoma of the lungs was found in this group. It should also be noted that in the experimental group the number of tumour nodules per mouse was higher than in the controls (1.7 as against 1.0).

As for hepatomas no statistically significant difference was found between the experimental and control groups, although in the group of females tumours occurred in greater numbers (22.5 per cent) than in the controls (5.5 per cent). In the males, hepatomas occurred less frequently in the experimental animals (21.0 per cent) than in the controls (35.0 per cent). As with lung adenomas, only one malignant hepatoma was discovered among the experimental animals and this showed infiltrative growth.

In summarizing the results of the experiments it may be concluded that an aqueous extract of barley grain attacked by *F. sporotrichioides* is blastogenic but only mildly so. It should be emphasized that we used aqueous extracts. On the one hand this type of extract is undoubtedly closer to actual conditions but on the other hand it may not provide complete results because of incomplete extraction of some blastogenic components. The identification of the blastogenic principle in the extract we examined required further investigations. The data already obtained, however, should be taken into account in analysing the results of a number of epidemiological investigations.

In the course of subsequent experiments the sera of mice treated with the extracts have been tested for presence of alpha-foetoprotein (AFP). Investi-

† The culture of the toxic mould *F. sporotrichioides* No. 63 was kindly supplied by Professor Sarkisov, to whom we are extremely grateful.

Table 1. Numbers of tumours in experimental and control mice

Sex	No. of mice surviving to one year of age*	Animals with tumours: Total tumours No.	Total tumours Per cent	Hepatomas No.	Hepatomas Per cent	Lung adenomas No.	Lung adenomas Per cent	No. per mouse
				Experimental mice				
Female	31	14†	45.1	7‡	22.5	11§	35.4	1.7
Male	19	8	42.1	4	21.0	7	36.8	1.1
Total	50	22	44.0	11	22.0	18	36.0	1.5
				Control mice				
Female	18	5‖	27.4	1	5.5	3	16.6	1.0
Male	17	6	35.2	6	35.2	1	5.8	1.0
Total	35	11	31.4	7	20.0	4	11.4	1.0
Reliability	Female		$X^2 = 0.830$ p 0.1		$X^2 = 1.436$ p 0.1		$X^2 = 0.975$ p 0.1	
	Male		$X^2 = 0.115$ p 0.1		$X^2 = 0.137$ p 0.1		$X^2 = 402$ p 0.05	
	Total		$X^2 = 1.272$ p 0.1		$X^2 = 0.071$ p 0.1		$X^2 = 5.132$ p 0.05	

* In animals which died before one year of age no tumours were found
† Including one undifferentiated carcinoma of unknown localization and one mammary-gland carcinoma
‡ One malignant hepatoma
§ One lung adenocarcinoma with metastases to other lobes
‖ One mammary-gland carcinoma

gation of its possible appearance in such animals is of special importance because in the literature, in some way, the belief prevails that hepatomas caused by mycotoxins are not associated with the appearance of the alpha-foetoprotein in the serum. This belief is based on the studies of Stanislawski-Birencwajs *et al.*[4] who stated that sera of hepatoma-bearing rats were positive for AFP when tumours were induced with dimethylnitrosoamine, several carcinogenic azo dyes, including 4-dimethylaminoazobenzene and 3-methyl-4-dimethylaminoazobenzene, but apparently not with aflatoxin B_1. Monjour and Mariage[5] were also unable to detect AFP in sera of rats with aflatoxin-induced hepatoma.

The exposure of mice started in October 1969. The total duration of the exposure of the experimental animals was equal to one year and experiments were finished in October 1970.

On 2 February 1971, a year and three months after beginning the experiment, samples of blood were collected from 49 mice, some of them had been receiving the extract and some were control mice. The sera of these experimental animals were examined by an ordinary gel-diffusion test. The examination was carried out under the supervision of Abelev and Perova. The results of the examination proved to be negative in all cases.

A little more than a month later, on 11 March 1971, 49 mice were once more examined for AFP. This time the immunoautoradiographic method

was used. This modified immunoautoradiographic method, developed by Elgort and Abelev[6], increases by 16–32 times the sensitivity of the gel-diffusion test. The method combines high sensitivity with absolute specificity and permits the detecting of this protein in the sera from patients with hepatomas giving a negative result in the ordinary gel-diffusion test. Elgort and Abelev have shown that in gel-diffusion AFP-negative sera from patients with hepatomas, more than 40 per cent were AFP positive when the immuno-autoradiographic method was used. The reactions were performed by Elgort to whom we are most thankful. The results of this investigation are presented in *Table 2.*

Table 2. Results of the test for alpha-foetoprotein

Group	No. of mice		Positive reaction		Negative reaction	
	Sex	No.	Liver tumour present	No liver tumour	Liver tumour present	No liver tumour
Experimental animals	♀	22	6	4	0	12
	♂	16	1	6	1	8
	Total	38	7	10	1	20
Controls	♀	8	1	1	0	6
	♂	3	1	1	0	1
	Total	11	2	2	0	7
Total		49	9	12	1	27
			21		28	

As will be seen from the data in the table, there was a significant association between tumour-bearing and AFP positivity. Of 10 tumour-bearing animals 9 were AFP positive, whereas of 39 non-tumour-bearing animals 12 were AFP positive. Similarly of 21 AFP positive animals 9 had tumours, whereas of 28 AFP negative animals only 1 had a tumour. These results indicate that a negative test for AFP, using the sensitive autoradiographic method, is usually associated with absence of tumour. It is of interest to speculate why many animals were positive for AFP in the absence of tumour. It may be in these hepatoma-susceptible mice that the presence of AFP indicates an early event in tumourogenesis, preceding the appearance of demonstrable tumour.

It is more difficult to relate AFP to the effect of treatment with *Fusarium* extract. No significant differences in the incidence of hepatomas in extract-treated mice and in a control group were observed. Thus the presence of AFP in the serum of most tumour-bearing animals of the treated group may not be necessarily related to the treatment, expecially since both of the tumour-bearing animals of the control group were also AFP positive. On the other hand, the AFP-negative reaction by such a sensitive technique as immunoautoradiography may have some value in indicating the possible absence of the hepatoma. Certainly our data needs further elaboration but in any case this data is challenging the studies which deny the AFP appearance in the hepatocarcinogenesis due to mycotoxins.

Special measurements had shown that the toxin contained in the grain infected with *Fusarium* is extracted with water as completely as with chloroform (the aqueous extract was extracted with chloroform and a comparative analysis made on the basis of intensity of fluorescence).

The purpose of the research described here was to determine certain physicochemical characteristics of 'fusariotoxin' and to establish whether it belongs to the same type as aflatoxins. With this in view, barley was infected with *F. sporotrichioides* and for control purposes with *Aspergillus flavus*. Use was also made of clean, uninfected grain in this experiment. We also had available a reference specimen of aflatoxin containing a mixture of aflatoxins B_1 (30 per cent) and G_1 (60 per cent), which was kindly supplied by the British Medical Research Council.

The experimental technique was as follows: 20 g of the grain was ground up in an electrically driven mill and extracted in packets of purified filter paper in a Soxhlet apparatus for 6 hours with distilled chloroform. The extract obtained was concentrated by driving off the solvent in a rotatory evaporator down to a volume of 5–8 ml. It was then subjected to thin-layer chromatography. A plate of silica gel was divided by a vertical stripe into two unequal parts. On the starting line in the wider part was placed 0.2 ml of the concentrated chloroform extract of the grain and in the narrower part 0.1 ml of the reference solution of aflatoxins in a concentration of 1×10^{-4} g ml^{-1}. Thus 10 µg of the reference sample of aflatoxins was applied to each plate as a control. The chromatogram was developed with a mixture of 2 per cent ethyl alcohol and 98 per cent distilled chloroform. The zones obtained by chromatography were examined for fluorescence under a mercury lamp with a UFS-2 filter allowing light to pass in the 3000–4000 Å band. Examination of the chromatograms showed that under the experimental conditions the reference preparations of aflatoxin produce one band with an intense dark blue-violet fluorescence, with $R_f = 0.2$. The chromatogram for the extract of grain infected with *Aspergillus flavus* showed a somewhat less bright blue-violet band than the control, but also with $R_f = 0.2$, and a faint greenish band with $R_f = 0.6$–0.7. On the chromatogram for the extract of grain infected with *Fusarium sporotrichioides* we observed a very faint bluish fluorescence in a band with $R_f = 0.2$ and a very intense bright blue-green band with $R_f = 0.7$. The chromatogram for the control extract of non-infected grain showed no fluorescence in the band with $R_f = 0.2$ while the band with $R_f = 0.7$ showed rather faint red fluorescence.

Thus, chromatographic examination confirmed that grain infected with *Aspergillus flavus* contains some substances with an R_f value and colour of fluorescence which coincide with the aflatoxins, and that grain infected with *F. sporotrichioides* also contains some fluorescent substances absent from 'clean' grain. However, both in their R_f value and in the colour of their fluorescence these substances were substantially different from the aflatoxins used as controls or produced by *Aspergillus flavus*.

In further researches the zones obtained on the plates were placed in funnels with Schott filters and washed with distilled chloroform until no fluorescence was seen. The volume of solvent used to wash out the zone was 50 ml. The solutions obtained were subjected to spectral fluorescence analysis.

We had worked out a method for quantitative determination of the group of aflatoxins contained in the reference solution used in our experiment. A series of reference solutions was set up with concentrations of 5×10^{-7}, 4×10^{-7}, 3×10^{-7}, 2×10^{-7}, 1×10^{-7} and 0.5×10^{-7} $g\,ml^{-1}$. The spectra of these solutions were recorded on a spectrographic device consisting of a monochromator with a diffraction grating of 1200 lines/mm, an FEU-18a photomultiplier, an amplifier and a PS-01 electronic recording potentiometer. The examinations were made in frozen solutions at the temperature of liquid nitrogen (a test tube was placed in a transparent-walled quartz Dewar flask, filled with liquid nitrogen). Under these conditions sensitivity in determining aflatoxin is several times as high as at room temperature. Fluorescence was initiated with a DKSS-1000 Xenon lamp with a UFS-2 filter allowing light in the 3000–4000 Å range to pass.

The spectrograms of the reference solutions were recorded in the 4000–4700 Å regions and a graph was plotted showing the relationship between the intensity of fluorescence at a wavelength of 4250 Å and the concentration of the solution. Investigations showed that the intensity of fluorescence at $\lambda = 4250$ Å measured over average background in the 4000–4700 Å region is proportional to the concentration at concentrations below 5×10^{-7} $g\,ml^{-1}$, and the graph can be used for determining the concentration of aflatoxins.

Our quantitative method was sensitive for 5×10^{-9} $g\,ml^{-1}$ of solution.

The spectrograms of the solutions as obtained by chromatography were recorded on the apparatus in the 4000–4700 Å waveband and the amount of aflatoxins was determined from the graph. In the solutions obtained from the aflatoxins applied to the plates as controls a concentration of 2×10^{-7} $g\,ml^{-1}$ was found in every case. Experimental points were also plotted on a graph. Calculation shows that if chromatography occurs without loss, 0.1 μg of aflatoxins applied to a plate as a control and removed from the plate with 50 ml of chloroform should produce a solution with a concentration of

$$\frac{10 \times 10^{-6}}{50} = 2 \times 10^{-7}\ g\,ml^{-1}$$

The concordance of the results obtained for the control aflatoxins on all plates with this figure within a limit of ± 10 per cent showed that there was a balance of the substance in chromatography and that our method of quantitative determination of the aflatoxin group was accurate. The amount of aflatoxins found on the basis of the spectrograms and the calibrated graph is as follows:

for uninfected grain—0
for grain infected with *Aspergillus flavus*—45 $\mu g\,g^{-1}$
for grain infected with *Fusarium sporotrichioides* below 10 $\mu g\,g^{-1}$

As already noted, the zone with intense blue-green fluorescence on the chromatogram for an extract of grain infected with *F. sporotrichioides* cannot be assigned to the aflatoxins since it differs substantially in R_f and in the colour of the fluorescence. Consequently the extract from this grain

contains some other fluorescent substances (possibly Fusariotoxin) which distinguish it from uninfected grain.

We attempted to determine what quantity of aflatoxin must be contained in an extract to ensure fluorescence of the intensity we observed. Calculations from the graph plotted for the aflatoxins showed that the grain would have to contain 3.5 μg of aflatoxin per g if the fluorescence of this extract were caused by the aflatoxins. Thus, if the fluorescence we observed belonged to the aflatoxins, 1 g of grain would contain 3.5 μg of aflatoxin, i.e. every animal in our mouse experiments would have been given 100 μg of aflatoxin. In such amounts a strong blastogenic effect could have appeared and yet the extract caused only a weak although statistically significant blastogenic effect. This result also shows that the toxin extracted from the grain infected with *Fusarium* obviously does not belong to the known aflatoxins.

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