Acta vet. scand. 1965, *6*, 224—233.

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# STUDIES ON ASPERGILLUS FUMIGATUS; TOXIN PRODUCTION BY DIFFERENT STRAINS AND SEROLOGICAL COMPARISON OF THE STRAINS

By
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Feedstuffs contaminated by growing Aspergillus fumigatus (AF) are known to cause deaths among calves (*Carll et al.* 1955) and pigs (*Shurawléw* 1962) and have been assumed to be a cause of haemorrhagic disease in poultry (*Forgacs et al.* 1962).

Toxic substances capable of inducing nervous signs and death in laboratory animals have been demonstrated in AF cultures using heat-killed spores (*Martins* 1929) or extracts from spores and mycelia obtained by treatment with fat solvents such as alcohol and ether (*Ceni et al.* 1902; *Bodin et al.* 1912; *Batelli* 1946).

*Henrici* (1939) liberated a toxin from AF by passing mycelial mats through a grinder and then compressing the material in a hydraulic press. The fluid obtained was lethal for rabbits, guinea pigs, mice, and chicks when given parenterally. The extract also had haemolytic activity and behaved as an antigen in the presence of homologous antiserum. Similar properties were described for an extract obtained by treatment of homogenized mycelia with McIlvaines' buffer (*Tilden et al.* 1957, 1961).

As a step in establishing the possible significance of AF toxins in causing mycotoxicosis in domestic animals, several strains isolated from various sources, chiefly feedstuffs, have been examined for their capacity for producing water-soluble toxins under different incubation conditions. The antigenic properties and serological relations between toxins of different strains have also been studied.

## MATERIALS AND METHODS

*Strains.* Nine strains of AF were isolated from different types of feedstuffs suspected of causing disturbances among animals. Another 5 strains were isolated from birds submitted for autopsy. The origins of the strains are listed in Table 1.

Table 1. The origin of 14 strains of A. fumigatus used in the present study.

| Strain | Source | | | Diagnosis |
|---|---|---|---|---|
| | Feed | Animal species | Organ | |
| 3 | Pig concentrate | | | |
| 938 | Pig concentrate | | | |
| 1854 | Ground grain | | | |
| 2144 | Pig concentrate | | | |
| 2833 | Oats | | | |
| 2834 | Barley | | | |
| 5580 | Ground mixed grain | | | |
| 6753 | Pig concentrate | | | |
| 7286 | Poultry concentrate | | | |
| 432 | | Capercaillie | Lung | Chronic mycotic pneumonia and aerocystitis |
| 6868 | | do. | do. | do. |
| 6869 | | Chick | do. | Acute mycotic bronchitis |
| 6870 | | Grouse | Musculature | Chronic mycotic ingluvitis with rupture of the crop and cellulitis |
| 6871 | | Chick | Lung | No signs of mycotic infection |

*Medium.* The strains were isolated on Sabouraud's agar and maintained on agar slants of that medium. Cultivation for toxin production was in yeast-extract broth consisting of Bacto-peptone Difco (1.5 %), yeast extract Difco (0.5 %), dextrose (3 %), and NaCl (0.5 %) in distilled water, pH 7.2.

*Toxin preparation.* AF cultures grown on Sabouraud's slants for 9 days at 20°C were transferred into 300 ml yeast-extract broth placed in flat Roux flasks. The mycelial mats growing on the surface of the media were collected, washed with 0.9 % NaCl solution, dried between filter paper and stored at —40°C for 2 to 4 days.

To every 100 g frozen mycelial material was added 75 ml of a 0.9 % NaCl solution and the mixture homogenized in a mixer at 11,000 r.p.m. The homogenate was stored at 4°C for 5 days, then centrifuged at 3,500 r.p.m. for 20 min., and the supernatant passed through Seitz

EK filters. One ml filtrate thus contains the substances from 1⅓ g wet mycelial material. The filtrates were stored at 4°C until use.

*Determination of toxicity.* The number of $LD_{50}$ for mice per ml filtrate for the different AF strains was calculated by *Kärbers* method. Groups of 6 mice belonging to the NMRI strain from the Naval Medical Research Institute, Bethesda, Ma, USA and weighing 16 to 18 g were injected with each of serial 10-fold dilutions of a filtrate. The volume of the inoculum was adjusted to 0.5 ml with 0.9 % NaCl solution. The mice were observed for 10 days.

*Haemolytic activity.* Sheep erythrocytes kept at 4°C for 4 days at the most and suspended in Alselver's solution were washed with 1/15 M phosphate buffer and diluted in the buffer to a 2 % suspension. Of this suspension, 0.5 ml was added to the strain filtrates in serial 2-fold dilutions beginning with 1:2 and made up to a volume of 0.5 ml with phosphate buffer. The haemolytic activity of the filtrates was read after incubation for 30 min. at 37°C and 90 min. at 20°C. The reciprocal of the highest dilution producing complete haemolysis has been taken as titer of the haemolytic activity and is expressed as the number of haemolytic units (HU) per ml.

*Immune serum.* Rabbits were injected intravenously at intervals of 3 or 4 days with filtrates from strains 938 and 2144 representing 5, 10, 20, 40, 80 and 120 $LD_{50}$ toxin. Serum was collected 10 days after the last injection. After heating at 56°C for 30 min. and the addition of merthiolate (1:10,000) the sera were stored at —20°C.

*Precipitation test.* Microtubes were used. A filtrate diluted 1:2 with a 0.9 % NaCl solution was layered over immune serum diluted 1:2 with normal serum. A grey-white layer appearing within 5 min. at the filtrate-serum interface has been considered as a positive reaction.

*Inhibition of haemolysis.* Serial dilutions of immune sera starting from 1/20 in volumes of 0.5 ml were mixed with 0.5 ml filtrate containing 4 HU. The mixtures were allowed to stand for 1 hr. at 20°C before 0.3 ml of the erythrocyte suspension was added. The results were read after 30 min. at 37°C and 90 min. at 20°C. The anti-haemolysin titer is expressed as reciprocal of the highest serum dilution causing complete inhibition.

*Protection test.* Immune sera prepared against filtrates of strains 938 and 2144 were injected intraperitoneally into mice weighing 16 to 18 g. Each mouse received 0.2 ml. About 20 hrs. later, the mice were injected intraperitoneally with filtrate from each strain containing 6 $LD_{50}$ toxin. Five mice were used for each strain filtrate and serum and 2 mice as controls for the filtrate alone. The mice were observed for 10 days.

## RESULTS

The haemolytic and toxic effects of filtrates of mycelial material from strain 938 obtained after incubation at 20°C for 6, 15 and 30 days declined as the incubation time was prolonged. The titer of haemolytic activity for example, dropped from 128 for the 6-days' filtrate to 8 HU/ml for the 30-days' filtrate. The corresponding decrease in toxicity was from 63 to 6 $LD_{50}$/ml.

The haemolytic and toxic activities of a filtrate obtained after incubation at 37°C for 6 days were the same as those obtained from cultures incubated at 20°C for the same time. After incubation at 37°C for 15 and 30 days, however, no haemolytic or toxic effect could be demonstrated in the filtrate.

Haemolytic activity and number of $LD_{50}$/ml for filtrates of strain 938 incubated at different temperatures and for different periods are given in Table 2. All haemolysin assays were done on one day with the same lot of sheep blood.

Table 2. Haemolytic activity and toxicity of filtrate from strain 938 of A. fumigatus.

| Incubation | | HU/ml | $LD_{50}$/ml (mice) |
|---|---|---|---|
| Temperature | Days | | |
| 20°C | 6 | 128 | 63 |
| | 15 | 128 | 40 |
| | 30 | 8 | 6 |
| 37°C | 6 | 128 | 63 |
| | 15 | $<2$ | $<1$ |
| | 30 | $<2$ | $<1$ |

The yeast-extract broths in which strain 938 had been cultured at 20°C for 15 and 30 days had haemolytic activities of 2 and 4 HU/ml respectively. Both cultures contained 3 $LD_{50}$ toxin/ml. Haemolytic or toxic activity could not be demonstrated in the yeast-extract broth in which strain 938 had been cultured at 37°C or at 20°C for only 6 days.

The haemolytic activity of filtrates obtained from 14 different AF strains after incubation at 20°C for 6 days varied from 64 to 1024 HU/ml and the toxicity varied from 25 to 398 $LD_{50}$/ml.

The haemolytic and toxic activities of filtrates from different strains are listed in Table 3. All haemolysin assays were done on one day with the same lot of sheep blood.

Table 3. Haemolytic activity and toxicity of filtrates from different strains of A. fumigatus.

| Strain | HU/ml | $LD_{50}$/ml (mice) |
|---|---|---|
| 3 | 128 | 63 |
| 432 | 64 | 25 |
| 938 | 256 | 100 |
| 1854 | 64 | 63 |
| 2144 | 64 | 100 |
| 2833 | 128 | 32 |
| 2834 | 128 | 32 |
| 5580 | 256 | 40 |
| 6753 | 64 | 40 |
| 6868 | 256 | 126 |
| 6869 | 1024 | 398 |
| 6870 | 1024 | 398 |
| 6871 | 512 | 200 |
| 7286 | 512 | 200 |

HU, haemolytic units

Table 4. Close immunological relationship between 14 strains of A. fumigatus.

| Sera / Filtrate | Precipitation | | Haemolysin inhibition | | Protection | | |
|---|---|---|---|---|---|---|---|
| | 938 | 2144 | 938 | 2144 | 938 | 2144 | Control |
| 3 | + | + | 160* | 160 | 0/5** | 0/5 | 2/2 |
| 432 | + | + | 80 | 80 | 0/5 | 0/5 | 2/2 |
| 938 | + | + | 40 | 40 | 1/5 | 0/5 | 2/2 |
| 1854 | + | + | 40 | 40 | 0/5 | 0/5 | 2/2 |
| 2144 | + | + | 80 | 160 | 0/5 | 0/5 | 2/2 |
| 2833 | + | + | 80 | 80 | 1/5 | 0/5 | 2/2 |
| 2834 | + | + | 80 | 80 | 1/5 | 1/5 | 2/2 |
| 5580 | + | + | 160 | 160 | 0/5 | 0/5 | 2/2 |
| 6753 | + | + | 160 | 160 | 0/5 | 0/5 | 2/2 |
| 6868 | + | + | 80 | 80 | 0/5 | 0/5 | 2/2 |
| 6869 | + | + | 80 | 80 | 1/5 | 0/5 | 2/2 |
| 6870 | + | + | 160 | 80 | 0/5 | 0/5 | 2/2 |
| 6871 | + | + | 160 | 160 | 0/5 | 0/5 | 2/2 |
| 7286 | + | + | 160 | 160 | 0/5 | 0/5 | 2/2 |

* Titer as reciprocal of the highest serum dilution.
** Number of dead mice/number of inoculated mice.

In the precipitation tests the filtrates from all the strains gave a positive reaction with sera against filtrates 938 and 2144. No precipitation occurred in control tests with normal serum.

Both antisera in dilutions from 1:40 to 1:160 inhibited the haemolytic effect of all the filtrates. In protection tests, the majority of mice pretreated with sera against filtrates 938 and 2144 survived the injection of 6 $LD_{50}$ of toxins from autologous and heterologous strains. The serum-injected mice which died generally survived longer (6—7 days) than the control mice (1—3 days).

The results of precipitation, haemolysin-inhibition and protection tests are summarized in Table 4.

Filtrates from strains 6870 and 6871 lost their haemolytic and toxic properties after being heated in a waterbath at 70°C for 10 min.

## DISCUSSION

Saline extraction of mycelial material from 14 strains of A. fumigatus (AF) gave filtrates with haemolytic and toxic properties. Qualitatively, these toxins seem to resemble the AF toxin described by *Henrici* (1939) and *Tilden et al.* (1961).

The filtrates were prepared by a uniform technique but there were differences in the haemolysin and toxin titers for the filtrates of different strains. *Tilden et al.* (1961) considered the composition of the culture medium, period of incubation, incubation temperature, and certain technical steps to be important for obtaining a highly potent toxin. In the present experiments, just as *Henrici* (1939) observed in his, a particular strain under apparently similar conditions could give on different occasions a filtrate with different degrees of haemolytic and toxic activities. Until the cause of those variations can be determined, there is no meaning in characterizing different AF strains as strong or weak toxin producers.

*Salvin* (1952) working with AF cultures grown on Sabouraud's agar, killed with formalin, and dried in cold acetone, reported that 1 $LD_{50}$ toxin for mice was obtained from 1.2 mg dried cell material but did not go into details concerning the toxic properties. The strains 6869 and 6870 which in the present series gave the highest toxin titers, had 1 $LD_{50}$ per 3⅓ mg wet mycelial material. It can, however, be assumed that with the technique used the extraction of toxin was incomplete.

The substance or substances responsible for the haemolytic and toxic properties can apparently be extracted from mycelial material only during a limited period of incubation. This period is much longer during incubation at 20°C than at 37°C. After extraction, however, haemolytic and toxic activities are maintained even after storage for several months in 4°C.

On the whole, a high haemolysin titer was accompanied by a high toxin titer. Unlike the results reported by *Tilden et al.* (1961), however, there was no definite correlation between the haemolytic and toxic activities. From this it seems as though the activities were associated with different chemical compounds even if these substances, according to *Rau et al.* (1961), appear in the same electrophoretic fraction.

The haemolytic and toxic activities in filtrates from strains 6870 and 6871 were thermolabile as were the toxins described by *Henrici* (1939) and *Tilden et al.* (1961).

The medium in which AF has been cultured is reported to contain a pyrogenic factor for rabbits (*Lucet* 1898). In a similar medium, *Bodin et al.* (1906) demonstrated two substances. One of these substances was ether-soluble and heat-stable and induced nervous signs and death in rabbits after intravenous injection. The other substance obtained from the medium by steam distillation was insoluble in ether and had a depressing effect on rabbits. Filtrates from peptonized broth in which AF had been grown exerted a lethal effect on rabbits and guinea pigs (*Batelli* 1946).

A release of haemolytic or toxic activity to the medium has not been observed by *Henrici* (1939). *Tilden et al.* (1961) considered that incubation at 37°C rather than 20°C enhanced toxin production. They were able to demonstrate haemolytic and toxic activity only after strong concentration of the medium in which AF was cultivated for 3 days at 37°C.

From the results of the present studies, it appears that more of the haemolytic and toxic activities are released to the medium after incubation of AF at 20°C than at 37°C. In fact with the methods used, haemolytic or toxic activity could not be detected after incubation at 37°C, even for a long time.

*Stanley* (1950) described a polysaccharide fraction in a mycelium extract of AF. This fraction had antigenic properties and low toxicity. A lipid fraction, causing monocytosis and multiple granulomas in guinea pigs was also obtained.

*Fukui et al.* (1961) prepared an antigen from formalin-killed mycelial material of AF by cryolysis. Antigenic properties were established by precipitation and complement-fixation tests but there were distinct reactions with normal serum as well. An alcoholic extract of the antigen was more specific and did not react in precipitation or complement-fixation tests with sera prepared against Cryptococcus neoformans and Candida albicans. An interesting result was that a serum prepared against the antigen gave a positive reaction, particularly in complement-fixation tests, with an antigen prepared from Penicillium chrysogenum. This result coincides with the observations of *Pepys et al.* (1959) that Aspergillus, Cladosporium and Penicillium have an antigen in common.

In precipitation tests, *Seeliger* (1958), observed that antigens from different species of aspergilli gave a group-specific reaction when tested against sera prepared with 20 other species of fungi. In agar-gel diffusion tests some type-specificity for different species of aspergilli could be detected (*Seeliger* 1955).

Using toxin from only one AF strain, both *Henrici* (1939) and *Tilden et al.* (1961) reported that a homologous immune serum had anti-haemolytic and anti-toxic effects. Type specificity of the toxin from Aspergillus flavus was demonstrated in precipitation tests.

The present study covered filtrates from 14 different AF strains isolated from feedstuffs and birds. Serological relationship between antigenic substances in all filtrates was demonstrated in precipitation tests. From the haemolysis-inhibition tests it appears that the haemolytic factor in the filtrates is immunologically uniform. The protection tests demonstrated immunological relationship between the toxic factors in the different filtrates.

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## SUMMARY

Saline extracts of mycelial material from 14 different strains of Aspergillus fumigatus showed haemolytic and toxic activities. These activities were present in filtrates obtained from mycelial material incubated at 20°C for 6, 15 and 30 days and at 37°C for 6 days. The haemolysin and toxin titers decreased with an increase in incubation time and at higher incubation temperatures.

The haemolytic and toxic activities could be inactivated by heating the filtrates at 70°C for 10 min.

The results of serological tests demonstrated immunological uniformity for the haemolytic and toxic factors.

## ZUSAMMENFASSUNG

*Studien von Aspergillus fumigatus; die Toxinproduktion in verschiedenen Stämmen und ein serologischer Vergleich der Stämme.*

Salz-Extrakte des Myceliummaterials von 14 verschiedenen Stämmen des Aspergillus fumigatus zeigten hämolytische und toxische Aktivität. Diese Aktivität war zugegen in Filtraten von dem Myceliummaterial bei 20°C in 6, 15 und 30 Tagen und bei 37°C in 6 Tagen inkubiert. Die Hämolysin- und Toxintiter fielen bei steigender Inkubationszeit und bei höherer Inkubationstemperatur.

Die hämolytische und toxische Aktivität konnte durch Erhitzen des Filtrates zu 70°C über 10 Minuten inaktiviert werden.

Die Ergebnisse der serologischen Teste zeigten immunologische Identität in bezug auf die hämolytischen und toxischen Faktoren.



## SAMMANFATTNING

*Studier av Aspergillus fumigatus; olika stammars toxinproduktion och serologisk jämförelse av stammarna.*

Filtrat från myceliematerial från 14 olika Aspergillus fumigatus stammar framställt genom extraktion med fysiol. koksaltlösning hade samtliga hämolytisk och toxisk aktivitet. Dessa aktiviteter kunde påvisas i filtrat från myceliematerial, som inkuberats i 20°C under 6, 15 och 30 dygn samt i 37°C under 6 dygn. Hämolysin- och toxintitrarna sjönk med längre inkuberingstid och högre inkuberingstemperatur.

Filtratens hämolytiska och toxiska verkan inaktiverades vid upphettning till 70°C under 10 minuter.

De serologiska undersökningarnas resultat utvisade immunologisk likformighet för de hämolytiska och toxiska faktorerna.


*(Received December 18, 1964).*