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STUDIES ON ASPERGILLUS FUMIGATUS; STABILITY OF HAEMOLYSIN AND TOXIN IN CRUDE FILTRATE

By
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Mycelial filtrates of Aspergillus fumigatus (AF) have proved to have toxic and haemolytic properties. When administered parenterally, they produce, as the most conspicuous patho-anatomical lesions, renal damage in several animal species (*Henrici* 1939, *Tilden et al.* 1961, 1963, *Rutqvist & Persson* 1966). In pigs, perirenal edema was, in addition, consistently observed (*Rutqvist & Persson*).

According to *Freeman* (1960), parenteral administration of AF toxin (prepared by *Tilden et al.* 1961) caused metabolic disturbances in female rats, in the form of hypercitricaemia and hypercalcaemia.

In in vitro experiments with AF toxin fractionated on DEAE-cellulose by *Wynston & Tilden* (1963), *Lee et al.* (1965) observed that a low toxin concentration stimulated respiration in renal tissue, whereas a high concentration impaired respiration and caused uncoupling of the oxidative phosphorylation.

It was shown in a previous investigation that, despite high toxicity on parenteral administration, no clinical symptoms or patho-anatomical changes appeared in mice, chicks or pigs when AF filtrates were given orally (*Rutqvist & Persson*). Consequently, the stability of the toxin and haemolysin in AF filtrates should be of interest. An account is given in this paper of a study of this property with respect to heat inactivation at different pH values. Studies were also made of the formation of toxoid under

the influence of formalin, as well as of the effect of various metals and potential enzyme inhibitors on, in the first place, the haemolysin.

MATERIAL AND METHODS

Apart from minor modifications, the methods were the same, in relevant parts, as those described earlier (*Rutqvist* 1965).

Strains. The AF strain 6869 was chosen for the inactivation experiments, and the AF strain 938 for the neutralization tests (*Rutqvist*).

Media. The strains were maintained at room temperature on agar slants of Sabouraud's agar, and were recultured once a month. Cultures for production of mycelial filtrates were made in fluid medium, consisting of proteose-peptone Difco (1.5 %), yeast extract Difco (0.5 %), dextrose (3 %) and NaCl (0.5 %) in distilled water, pH 7.2.

Filtrate preparation. Strain cultures grown for 7 days at 20°C on Sabouraud's agar in agar slants were suspended with 10 ml of proteose-peptone broth per tube, and 1 ml was transferred to 300 ml of fluid medium in Roux flasks. The mycelial mats, which after 3 days' incubation at 37°C had grown on the surface of the medium, were washed on harvesting with sterile, distilled water, dried on filter paper and kept at — 20°C for 2—4 days.

To 100 g of frozen mycelial material was added 75 ml of sterile distilled water, and the mixture was homogenized in a mixer at 11,000 r. p. m. The homogenate was stored at 4°C for 5 days and centrifuged at 2,500 × g for 20 min., after which the supernatant was filtered through a Seitz EK filter. The filtrate was frozen, and stored at — 20°C.

Two filtrates prepared from strain 6869 were used for the experiments. In one of them, used in heat inactivation at various pH, the titre for LD_{50} in mice (denoted here as MLD_{50}) and the haemolytic unit (HU) were $10^{-2.6}$ and 2^{-10} ml, respectively. In the other preparation from strain 6869, used for toxoid preparation, and in the filtrate preparation from strain 938, the corresponding titres were of the same size in both filtrates, and amounted to $10^{-2.2}$ and 2^{-9} ml, respectively. The titres comprised the mean of 2—6 determinations per filtrate. The two filtrates from strain 6869 had a pH of 6.7. The pH of the strain 938 filtrate was 7.0.

Toxicity. The MLD_{50} titre was calculated by Kärber's formula. Groups of 10 mice belonging to the NMRI strain (Naval Medical Research Institute, Bethesda, Md., USA), weighing 16—18 g, were inoculated intraperitoneally with each of a series of 10-fold dilutions of the filtrate. The dilution fluid was 0.9 % NaCl solution. The volume of the inoculum was 0.5 ml per mouse. The mice were kept under observation for 10 days.

Haemolytic activity. Sheep erythrocytes in Alsever's fluid, kept at 4°C for maximally 4 days, were washed with 1/10 M veronal buffer, pH 7, and diluted in the buffer to a 2 % suspension. Of this suspension, 0.5 ml was added to 0.5 ml of the strain filtrate in 2-fold serial dilutions with 0.9 % NaCl. The haemolytic activity was read after incubation for 30 min. at 37°C and 90 min. at 20°C. The smallest quantity of filtrate producing complete haemolysis was taken as the titre of haemolytic activity, and expressed as 1 haemolytic unit (HU).

Immune serum. Antiserum to strain 938 filtrate was obtained from rabbits which, at 3—4-day intervals, had been injected intravenously with a filtrate containing 5, 10, 20, 40, 80 and 120 MLD_{50} toxin. The serum was collected 10 days after the last injection.

Strain 6869 filtrate antiserum was prepared by using formalin-inactivated filtrate as antigen. Inactivation took place by incubation at 37°C for 5 days of a formalin-filtrate mixture with a 6×10^{-2} M concentration of formalin. Five ml of formalin-inactivated antigen was mixed with 5 ml of Bacto adjuvant complete Freund, Difco. Five ml of the mixture was inoculated subcutaneously in rabbits on two occasions, at an interval of 14 days. The serum was collected 14 days after the last injection.

After heating at 56°C for 30 min. and addition of merthiolate (1:10,000), the sera were stored at 4°C.

Normal serum. Normal serum was obtained from a healthy rabbit, and stored at 4°C after heating at 56°C for 30 min., and addition of merthiolate (1:10,000).

Inhibition of haemolysis. Serial dilutions 1:20, 1:40, etc. of immune sera and normal serum with 0.9 % NaCl solution in volumes of 0.5 ml were mixed with 0.5 ml of filtrate dilution containing 4 HU. The mixture was allowed to stand for 1 hr. at 20°C, after which 0.3 ml of erythrocyte suspension was added. The results were read after incubation for 30 min. at 37°C and for 90 min. at 20°C.

Neutralization experiments. To each dilution of immune sera and normal serum diluted 2-fold with 0.9 % NaCl was added filtrate from strain 938 diluted with 0.9 % NaCl so that 0.6 ml of the filtrate-serum mixture contained 3.5 MLD_{50} toxin. The proportions between filtrate and serum dilutions were such that each of 10 mice per serum dilution was inoculated intraperitoneally with 0.5 ml of serum dilution and 0.1 ml of filtrate dilution. Before inoculation was performed, the filtrate-serum mixtures had been kept for 1 hr. at 20°C and for 20 hrs. at 4°C. The mice were kept under observation for 10 days.

pH determination. All pH determinations were made with a glass electrode, using a type 28 pH meter (Radiometer, Copenhagen, Denmark).

Chemical substances. All chemicals used in the experiments were of analytical grade.

Table 1. Inactivation of toxin and haemolysin in crude filtrate at 60°C (pH 6.7) and 40°C (pH 2.5 and 9.5). Aspergillus fumigatus strain 6869. Residual titres in ml of toxin (MLD_{50}) and haemolysin (HU).

Duration of heating min.	60°C pH 6.7		40°C pH 2.5		40°C pH 9.5		Temperature control 40°C pH 6.7		pH control 1 4°C pH 2.5		pH control 2 4°C pH 9.5	
	MLD_{50}	HU	MLD_{50}	HU	MLD_{50}	HU	MLD_{50}	HU	MLD_{50}	HU	MLD_{50}	HU
0	$10^{-2.6}$	2^{-10}	$10^{-2.6}$	2^{-10}	$10^{-2.6}$	2^{-10}	$10^{-2.6}$	2^{-10}	$10^{-2.5}$	2^{-10}	$10^{-2.7}$	2^{-10}
10	—*)	2^{-9}	$10^{-1.9}$	2^{-8}	—	—	—	—	—	—	—	—
20	—	2^{-8}	$10^{-1.6}$	2^{-6}	—	—	—	—	—	—	—	—
30	—	2^{-7}	$10^{-0.8}$	2^{-3}	—	2^{-8}	—	2^{-10}	$10^{-2.6}$	2^{-10}	—	—
45	—	2^{-4}	$10^{-0.1}$	$>2^{-1}$	—	2^{-6}	—	—	—	—	—	—
60	$10^{-1.7}$	2^{-1}	$>10^{0}$		$10^{-2.2}$	2^{-5}	$10^{-2.7}$	2^{-10}	$10^{-2.7}$	2^{-10}	$10^{-2.7}$	2^{-10}
75	—	$>2^{-1}$			$10^{-1.8}$	2^{-4}	—	2^{-10}	—	2^{-10}	—	2^{-10}
90	$10^{-0.8}$				$10^{-1.7}$	2^{-3}	$10^{-2.5}$	2^{-10}	$10^{-2.6}$	2^{-9}	$10^{-2.5}$	2^{-10}
120	$10^{-0.8}$				$10^{-1.4}$	2^{-1}	—	—	—	—	$10^{-2.6}$	2^{-10}
150	$10^{-0.7}$				$10^{-1.2}$	$>2^{-1}$	$10^{-2.7}$	2^{-10}	—	—	$10^{-2.8}$	2^{-10}

*) not investigated.

EXPERIMENTAL AND RESULTS

Influence of heat on toxin and haemolysin at various pH

The stability of the haemolysin and toxin in a crude filtrate of strain 6869 was studied after heating for 10—150 min. This was done both at 60°C at a pH of 6.7, and at 40°C at pH values of 2.5 and 9.5, respectively. The temperatures and pH values of 2.5 and 9.5 were chosen on the basis of results of preliminary experiments. These showed that an increase in the stated temperatures of 5°C at the different pH values produced a disappearance of haemolytic activity within 30 min., whereas a decrease of 5°C resulted in persisting haemolytic activity after 2 hrs. heat treatment. In filtrates heated at 40°C, a reduction in pH below 2.5 and a rise above 9.5 by 0.5 pH caused haemolytic activity to disappear within 30 min. When, on the other hand, these pH values were raised and lowered, respectively, by 0.5 pH unit, the activity persisted after 2 hrs. heating.

The pH of the filtrates was adjusted with 2 N-HCl and 2 N-NaOH. Heating was done in a water bath, the filtrate being heated in 5-ml portions in thin-walled, stoppered glass tubes. Immediately after the tubes had been removed from the water bath, they were cooled under running cold water, and the pH was adjusted to 6.7. They were then kept at 4°C until the activity was determined, which generally took place within 2 hrs.

The results of the inactivation experiments can be inferred from Table 1. In Figs. 1—3, the decrease in the titres of the toxic and the haemolytic activity is plotted against time.

The inactivation curves for toxin and haemolysin showed a wide divergence, with better stability of the toxin when the filtrate was heated at 60°C with a pH of 6.7 and at 40°C with a pH of 9.5 (Figs. 1 and 3). Heating at 40°C and pH 2.5 resulted, as can be seen in Table 1, in rapid inactivation of both haemolysin and toxin. In this case, it seems possible for both inactivation curves to be represented by the same straight line (Fig. 2). Merely heating of the filtrate at 40°C at a pH of 6.7, as well as storing in the refrigerator after adjusting the pH to 2.5 and 9.5, respectively, with neutralization to pH 6.7 after the times given in Table 1, seemed to have no effect on the activity of either the haemolysin or the toxin (Table 1).

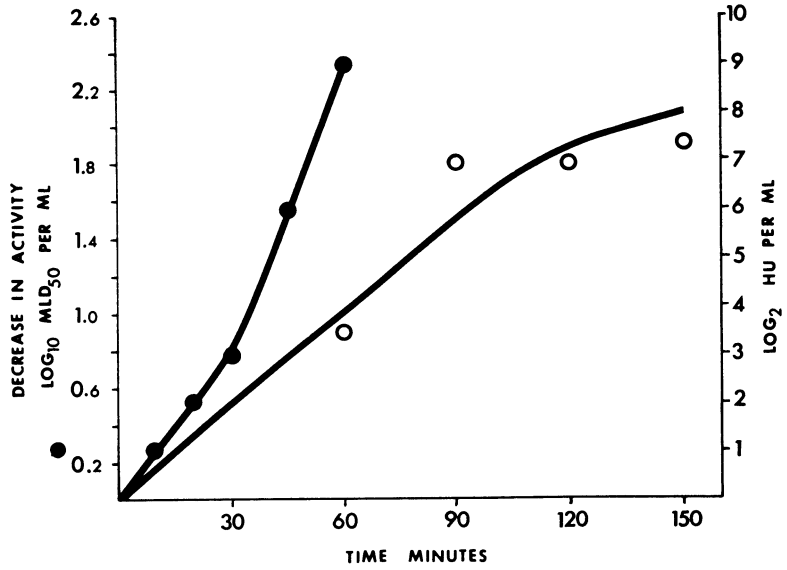

Figure 1. Inactivation at 60°C and pH 6.7 of haemolysin and toxin. Aspergillus fumigatus strain 6869.

● ——— ● HU.
o ——— o MLD_{50}.

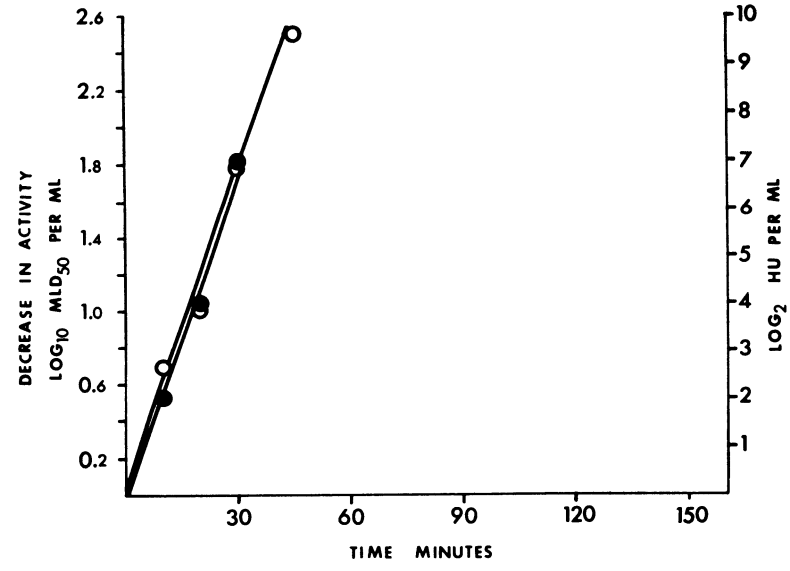

Figure 2. Inactivation at 40°C and pH 2.5 of haemolysin and toxin. Aspergillus fumigatus strain 6869.

● ——— ● HU.
o ——— o MLD_{50}.

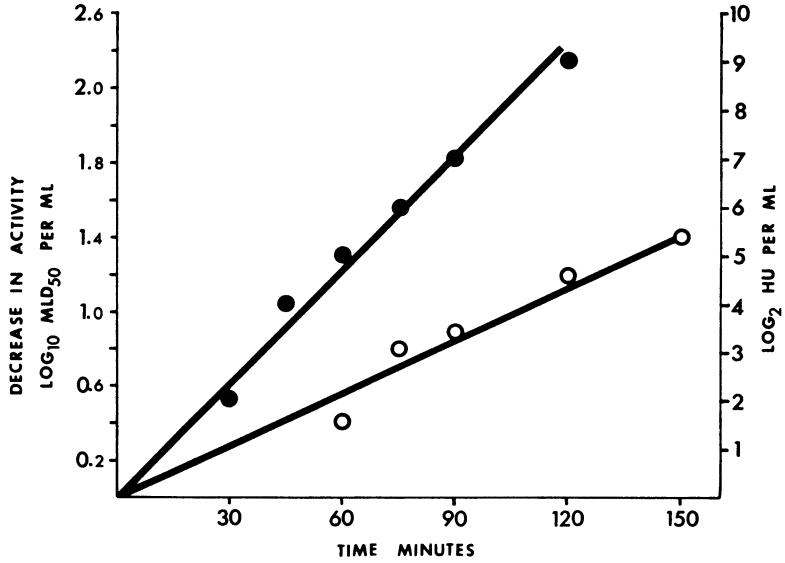

Figure 3. Inactiviation at 40°C and pH 9.5 of haemolysin and toxin. Aspergillus fumigatus strain 6869.

● ——— ● HU.
o ——— o MLD_{50}.

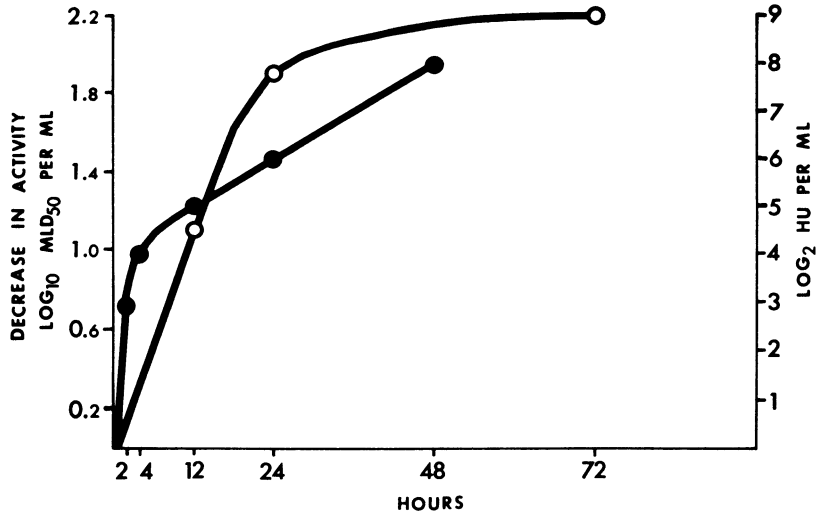

Figure 4. Formalin inactivation at 37°C and pH 6.7 of haemolysin and toxin. Aspergillus fumigatus strain 6869.

● ——— ● HU.
o ——— o MLD_{50}.

Inactivation of toxin and haemolysin by formalin

In order to investigate if toxin and haemolysin could be transformed into toxoids, experiments with formalin treatment and neutralization tests were performed.

Table 2. Inactivation at 37°C (pH 6.7) of toxin and haemolysin in crude filtrate with addition of formalin to a final concentration of 6×10^{-2}M. Aspergillus fumigatus strain 6869. Residual titres in ml of toxin (MLD_{50}) and haemolysin (HU).

Strain	Activity	Duration of formalin treatment, hours							
		0	2	4	12	24	48	72	96
6869	MLD_{50}	$10^{-2.2}$	—*)	—	$10^{-1.1}$	$10^{-0.3}$	—	10^{0}	$>10^{0}$
	HU	2^{-9}	2^{-6}	2^{-5}	2^{-4}	2^{-3}	2^{-1}	$>2^{-1}$	
Temperature Control	MLD_{50}	$10^{-2.2}$	—	—	—	—	—	—	$10^{-2.3}$
	HU	2^{-9}	—	—	2^{-9}	—	—	—	2^{-8}

*) not investigated.

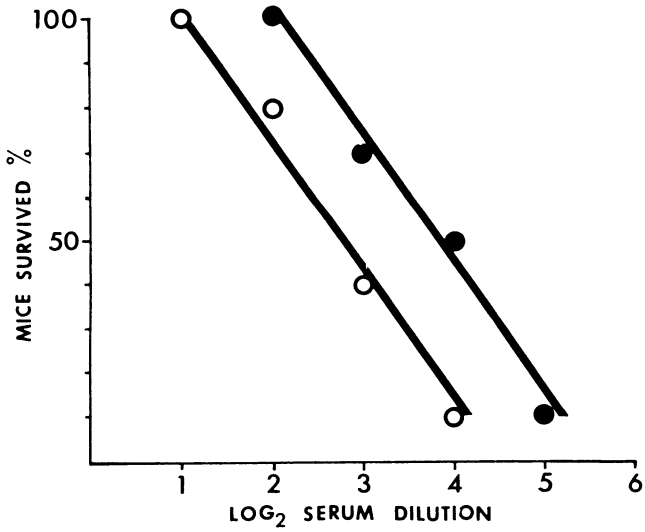

Figure 5. Neutralizing effects of immune sera prepared against formalinized (strain 6869) and non-formalinized (strain 938) antigens from Aspergillus fumigatus.

●——— ——● 6869 immune serum.

o——— ——o 938 immune serum.

In filtrate with pH 6.7 and a 6×10^{-2} M concentration of formalin, incubated in a thermostat at 37°C, haemolytic activity could not be demonstrated after 72 hrs. incubation, and after 96 hrs. no toxic activity was to be found (Table 2). The greater part of the activity of both haemolysin and toxin disappeared during the first 24 hrs. of incubation. In Fig. 4, where the titres of decrease in haemolysin and toxin activities are plotted against time, the resulting inactivation curves are shown.

Neutralization of toxin and haemolysin by antisera

It is evident from Fig. 5 that immune sera against formalinized (strain 6869) and non-formalinized (strain 938) filtrates had a toxin-neutralizing effect. A better neutralizing effect could be observed in immune serum prepared against formalinized filtrate to which adjuvant had been added.

Normal serum in 1:2 dilution had no toxin-neutralizing effect.

Both immune sera inhibited haemolysis in a dilution of 1:40, whereas normal serum in a dilution of 1:20 had no inhibitory effect on the haemolytic activity.

Influence of some metal ions and potential enzyme inhibitors on haemolysin in crude filtrate

In work with filtrate preparations preserved with merthiolate and stored at 4°C, it had been observed that the haemolytic activity gradually decreased and after some time disappeared. The toxic activity, however, was apparently not influenced.

The disappearance of haemolytic activity caused by merthiolate made it of interest to investigate the influence on the haemolysin of other mercury compounds and some common heavy metals and potential enzyme inhibitors.

The chemical substances listed in Table 3 were allowed to act on the filtrate for 60 min. in a thermostat at 37°C, after which the haemolytic activity was determined. The results are given in Table 3.

No haemolytic activity was demonstrated in filtrates with a 10^{-2}M and 10^{-3}M concentration of mercury. A 10^{-4}M concentration of Hg also had a marked immediate effect, and the haemolysin was completely inactivated after 60 min. incubation at 37°C. With an Hg content of 10^{-5}M, full haemolytic activity

Table 3. Influence of some metal ions and potential enzyme inhibitors on haemolysin in crude filtrate. Aspergillus fumigatus strain 6869.

Substance	Molarity	pH	Haemolysin (HU) titre in ml after incubation at 37°C	
			0 min.	60 min.
Filtrate		6.7	2^{-9}	2^{-9}
Zn^{++}[1])	10^{-2}	5.8		2^{-9}
Cu^{++}	10^{-2}	5.0		2^{-9}
Co^{++}	10^{-2}	6.3		2^{-9}
Fe^{++}	10^{-2}	6.2	2^{-9}	2^{-8}
Ca^{++}	10^{-2}	6.7		2^{-9}
Hg^{++}	10^{-2}	5.9	$>2^{-1}$	
„	10^{-3}	6.7	$>2^{-1}$	
„	10^{-4}	6.7	2^{-2}	$>2^{-1}$
„	10^{-5}	6.7		2^{-9}
EDTA[2])	10^{-2}	6.3		2^{-9}
Potassium cyanide	10^{-2}	7.8		2^{-9}
Iodoacetic acid	10^{-2}	6.3		2^{-9}
L-cysteine	10^{-2}	5.7		2^{-9}
Glutathione	10^{-2}	5.8		2^{-9}

[1]) The metals were solutions of $ZnSO_4$, $CuSO_4$, $CoCl_2$, $FeSO_4$, $CaCl_2$ and $HgCl_2$.

[2]) Disodium salt of ethylene diamine tetraacetic acid.

was noted. Inactivation of the haemolysin was demonstrable directly after the addition of mercury to the filtrate.

The other substances tested produced no definite change in the haemolytic activity.

Reactivation of mercury-inactivated haemolysin

L-cysteine or glutathione to a final concentration of 2.5×10^{-3}, 5×10^{-3} and 10^{-2}M was added to filtrates with a 10^{-3}M final concentration of mercury chloride and p-chloromercuribenzoate, respectively. Reactivation of the mercury-inactivated haemolysin was then demonstrable (Table 4). Reactivation was complete in filtrate mixtures with a 10^{-2}M final concentration of L-cysteine or glutathione.

Table 4. Influence of L-cysteine and glutathione on the haemolysin-inhibiting effect of mercury compounds. Aspergillus fumigatus strain 6869.

Inhibitor	Molarity	Haemolysin (HU) titre in ml							
		L-cysteine molarity				Glutathione molarity			
		0	2.5×10^{-3}	5×10^{-3}	10^{-2}	0	2.5×10^{-3}	5×10^{-3}	10^{-2}
None		2^{-9}				2^{-9}			
Mercury chloride	10^{-3}	$>2^{-1}$	$>2^{-1}$	2^{-7}	2^{-9}	$>2^{-1}$	2^{-6}	2^{-8}	2^{-9}
p-Chloromercuri-benzoate*)	10^{-3}	$>2^{-1}$	$>2^{-1}$	2^{-4}	2^{-9}	$>2^{-1}$	2^{-5}	2^{-8}	2^{-9}

*) Sodium salt of p-chloromercuribenzoic acid.

Toxic activity in mercury-treated filtrate

Addition of p-chloromercuribenzoate to a final concentration of 10^{-3}M to AF filtrate with pH 6.7 appeared to have no definite effect on the toxic activity of the filtrate after keeping the filtrate-mercury mixture for 1 hr. at 37°C, and subsequent storage for 7 days at 4°C. After storage, the haemolysin was completely reactivated on addition of L-cysteine to a final concentration of 10^{-2}M (Table 5).

Table 5. Haemolytic and toxic activity and haemolysin-reactivating ability of L-cysteine in mercury-treated filtrate stored at 4°C. Aspergillus fumigatus strain 6869.

Inhibitor	Reactivator	Haemolysin (HU) titre in ml days		Toxin (MLD_{50}) titre in ml days	
		0	7	0	7
None	None	2^{-9}		$10^{-2.2}$	
p-Chloromer-curibenzoate 10^{-3}M	None	$>2^{-1}$	$>2^{-1}$	$10^{-2.3}$	$10^{-2.1}$
„	L-cysteine 10^{-2}M	2^{-9}	2^{-9}		

DISCUSSION

A distinct difference was present between the stability of the haemolysin and that of the toxin, the toxin being the more stable. This was demonstrated when the filtrate with a pH of 6.7 was heated at 60°C, and when the filtrate with a pH of 9.5 was heated at 40°C. This finding is in agreement with that of *Tilden et al.* (1961, 1963), and would indicate that the haemolysin and the toxin are different principles. Although *Wynston & Tilden* (1963), using chromatography, could not completely separate haemolysin and toxin, they found the greatest haemolytic and toxic activity in different fractions. The dermo-necrotic effect of the AF filtrate in the rabbit proved to be bound to the haemolysin (*Tilden et al.* 1963).

Rapid inactivation of both haemolysin and toxin was obtained by a pH of 2.5 at 40°C. This suggests that gastric juice may have an inactivating effect on the toxin and may explain the absence of a toxic action of the filtrate when given orally, despite its high toxic activity when administered parenterally (*Rutqvist & Persson* 1966).

Immediate blocking of the activity of the haemolysin by mercury compounds, as well as the reactivating effect of thiol compounds, are of considerable interest. Like pneumococcal haemolysin (*Shwachman et al.* 1934) and the haemolysin from cobra venom (*De* 1940a, b), the AF haemolysin shows in this respect a striking parallelism with several enzymes whose effect is considered to depend on active sulphydryl groups. With various mercury compounds they can form mercaptides which are, however, easily reversible under the action of thiols with greater affinity to the inhibiting substance (*Barron* 1951, *Boyer* 1959).

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SUMMARY

The haemolysin and toxin in mycelial filtrates of Aspergillus fumigatus showed a distinct difference in stability when the filtrate was heated at 60°C and pH 6.7, as well as at 40°C and pH 9.5, the toxin being more stable. When the filtrate was heated at 40°C and pH 2.5, a rapid inactivation of both haemolysin and toxin occurred.

No haemolytic or toxic activity could be demonstrated in a filtrate with a 6×10^{-2}M concentration of formalin after incubation at 37°C for 3 and 4 days, respectively. Serum prepared with formalin-treated filtrate as antigen had a haemolysin- and toxin-neutralizing effect.

When mercury chloride or p-chloromercuribenzoate in a final concentration of 10^{-3}M was added to the filtrate, immediate blocking of the haemolytic activity, but not of the toxic, was obtained.

Haemolysin inactivated by mercury in a final concentration of 10^{-3}M was reactivated under the influence of L-cysteine or glutathione in a final concentration of 10^{-2}M.

ZUSAMMENFASSUNG

Studien über Aspergillus fumigatus; Hämolysin und Toxin — ihre Stabilität in Rohfiltrat.

In Myzelienfiltraten von Aspergillus fumigatus zeigten Hämolysin und Toxin deutlich voneinander abweichende Thermostabilität. Das Toxin erwies sich stabiler als das Hämolysin, falls Filtrate mit pH 6.7 auf 60°C oder mit pH 9.5 auf 40°C erhitzt wurden.

Durch Erhitzung von Filtraten mit pH 2.5 auf 40°C wurden das Hämolysin und das Toxin schnell inaktiviert.

Nach 3—4-tägiger Wärmebehandlung von 6×10^{-2}M Formalin enthaltenden Filtraten bei 37°C konnte weder hämolytische noch toxische Aktivität nachgewiesen werden. Serum, hergestellt mit durch Formalin inaktiviertem Filtrat, neutralisierte das Hämolysin und das Toxin.

Eine unmittelbare Blockierung der hämolytischen Aktivität der Filtrate wurde durch den Zusatz von Quecksilberchlorid oder p-Chlormerkuribenzoat in einer Endkonzentration von 10^{-3}M erreicht. Die toxische Aktivität wurde dadurch nicht beeinflusst. Durch Zusatz von L-Zystein oder Glutathion in der Endkonzentration von 10^{-2}M wurde das Hämolysin vollständig reaktiviert.

SAMMANFATTNING

Studier av Aspergillus fumigatus; stabilitet hos hämolysin och toxin i råfiltrat.

Hämolysin och toxin i myceliefiltrat från Aspergillus fumigatus visade tydligt divergerande stabilitet när filtratet upphettades vid 60°C, pH 6.7 och vid 40°C, pH 9.5 varvid toxinet visade bättre stabilitet än hämolysinet. När filtratet upphettades vid 40°C, pH 2.5, påvisades en snabb inaktivering av hämolysin och toxin.

Efter inkubering i 37°C under tre respektive fyra dygn kunde i filtrat med 6×10^{-2}M koncentration av formalin, hämolytisk resp. toxisk aktivitet icke påvisas. Serum framställt med formalinbehandlat filtrat som antigen hade hämolysin- och toxinneutraliserande verkan.

Omedelbar blockering av den hämolytiska men ej den toxiska aktiviteten erhölls när kvicksilverklorid eller p-klormerkuribenzoat i 10^{-3}M slutkoncentration sattes till filtratet.

Av kvicksilver i 10^{-3}M slutkoncentration inaktiverat hämolysin reaktiverades fullständigt under inverkan av L-cystein eller glutation i 10^{-2}M slutkoncentration.

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