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Fungal antigens as a source of sensitization and respiratory disease in Scottish maltworkers

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Summary

Mycological and serological studies were carried out as part of a survey of respiratory disease in Scottish maltworkers. 70% of stained sputum smears from 574 workers showed the presence of higher plant cells and/or mycelia, and the spores of common environmental fungi. *Penicillium* spp. (90%), *Rhizopus stolonifer* (48%) and yeasts (53%) were the dominant fungi in 699 sputum cultures, and showed a similar proportional distribution in 327 samples of grain, malt, culms and dusts from fifty-six maltings.

57% of 711 men were serologically positive for fungi, 22% for *Aspergillus fumigatus*, 20% for *A. clavatus*, 10% for *A. niger*, 16% for *Cladosporium herbarum* and over 3% for *Rhizopus stolonifer*. 6% of 132 men were positive for *Penicillium cyclopium*. No precipitating antibodies to antigens from *Alternaria tenuis*, *Aureobasidium pullulans*, *Candida albicans*, *Geotrichum candidum*, *Rhodotorula glutinis* or *Trichoderma viride* were detected in tests of forty sera.

Sera from the 5·2% of men with symptoms of extrinsic allergic alveolitis showed increased reactivity to mycelial antigens from *Aspergillus clavatus*. The fungus was cultured from 21% of maltings, 7% of all environmental samples and from the sputa of 8% of maltworkers.

Introduction

The occurrence of respiratory disease, sometimes severe, among Scottish maltworkers was reported by Riddle *et al.* (1968) and Channell *et al.* (1969) when describing four cases of maltworker's lung. The disease was shown to be an example of extrinsic allergic alveolitis (EAA) caused by hypersensitivity to inhaled organic dust containing propagules of *Aspergillus clavatus* Desmazières. A subsequent survey of fifty-six maltings throughout Scotland in 1972 (Grant *et al.*, 1976) showed that the industry had an incidence of 5·2% for the disease. The mycological and serological studies carried out during the survey confirmed that *A. clavatus* was a major source of sensitizing and disease-inducing antigen, and these studies are described in the following paper.

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The malting process in relation to disease

The process is summarized in Fig. 1. Intake barley, which is of variable moisture content on receipt, is dried in kilns and brought to about 11% moisture content. The grain is then screened, and unwanted fractions such as small grains, chaff and dust are removed, bagged, and sold for cattle feed. Screened grain, after storage for some months, it is then steeped for about 36 hr to raise its moisture content to between 40% and 50%, before being spread in layers 10 to 15 cm deep on open malting floors to allow a period of germination and growth of 7–8 days. Alternatively, the grain may be poured into boxes (Saladin) in layers of about 1·6 m deep, or introduced into enclosed malting systems (Domalt or Drum). In the Domalt process, the grain moves slowly on a conveyor belt during malting; in the Drum method it lies in a drum which slowly rotates. The common objective of each process is to provide optimal conditions of aeration, temperature and humidity for germination and growth. In all but open-floor malting, turning of grain is achieved by some mechanical means.

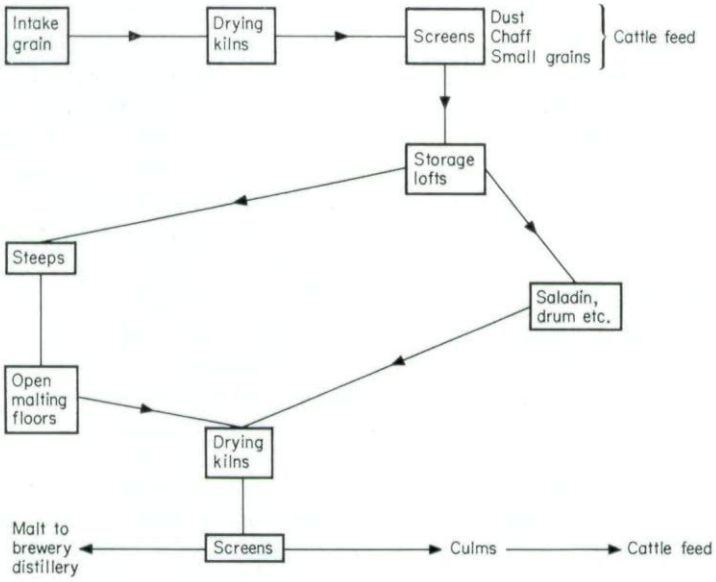

Fig. 1. The malting process.

Organic dusts of immunogenic and allergenic significance to the workforce result from the release of microbial contaminants, many of which proliferate during the malting process. Dusts are effectively contained within the enclosed systems, but are freely released from Saladin boxes and open floors. Grain is turned on open floors by shovel or mechanical plough, and although maltworkers may move from process to process even in the same maltings, it is not surprising that 6·8% of men working predominantly on open floors, and 6·2% of those working on Saladin malting, had allergic alveolitis, whereas only 1·1% of men on enclosed processes were affected (Grant *et al.*, 1976).

After germination and growth, malt is moved to kilns and dried to about 5% moisture content by raising the temperature to 82°C over 48 hr. It is then passed

through vibratory screens to remove rootlets (culms), which are used for cattle feed, and is finally stored in silos. Large quantities of dust are released during these processes.

Materials and methods

Environmental samples

Medical teams visited each of fifty-six maltings for the purpose of supervising the filling-in of MRC questionnaires on respiratory symptoms, determining skin sensitivities to allergens, obtaining samples of blood and sputum, and carrying out tests of respiratory function. They were provided with environmental packs, one for each maltings, containing colour-coded, sterile, 25 ml polystyrene screw-capped vials for the collection of samples of intake grain, malt from open floors or other processes, culms and dusts from screening machinery. A sterile 9·0 cm disposable Petri dish, containing 2% Oxoid malt extract agar, was included in each pack for exposure as a settle plate for 2 min in the vicinity of malt being turned.

As the objective of the mycological part of the survey was to determine the nature of the mesophilic fungal flora on the surfaces of samples with which the workers were in contact, no attempt was made to distinguish between the locations of propagules by the use of surface sterilants or heat before plating. All samples were plated on 2% Oxoid malt agar. Duplicate sets of five grains, from samples of intake grain and malt, were plated out by means of heat-sterilized forceps. Culms were lightly sprinkled onto agar surfaces directly from collection vials. Dusts were blown, by means of a jet of compressed air delivered through the inlet tube of a modified vial lid, and released through an outlet tube to the agar surface via a filter funnel. This ensured an even distribution of particles. All plates were incubated at 26°C in darkness. A total of 327 samples was processed.

Sputum samples

Slide smears were made from each of 699 sputa by means of a heat-sterilized inoculating loop, and were heat-fixed. After staining by the Gomori–Grocott methenamine–silver nitrate method (Grocott, 1955), 574 smears were found to be suitable for detailed microscopic examination. The entire remainder of each sputum sample was cultured at 26°C on 2% Oxoid malt agar, in darkness, for 7 to 14 days. Sputa were classified as watery or mucoid/mucopurulent according to consistency and cellular content.

Immunology

Fungal extracts for skin and *in vitro* testing were prepared by growing environmental isolates of selected species, at 26°C, in 400 ml aliquots of either Oxoid Sabouraud dextrose broth or Oxoid Czapek Dox liquid medium in 1 litre conical flasks on a rotary shaker for periods of 3 to 5 weeks. The contents of the flasks were processed to give culture filtrate (CF) and mycelial (M) extracts according to the flow chart (Fig. 2).

Prick tests were carried out to determine the incidence of type I hypersensitivity responses to allergens. Common allergens of commercial origin included grass pollen, *Aspergillus fumigatus*, *Dermatophagoides pteronyssinus* and control extracts. Extracts made from the mycelia of *Aspergillus clavatus*, *A. niger*, *Rhizopus stolonifer*, *Penicillium cyclopium*, *Cladosporium herbarum* and *Micropolyspora faeni*, and formulated in

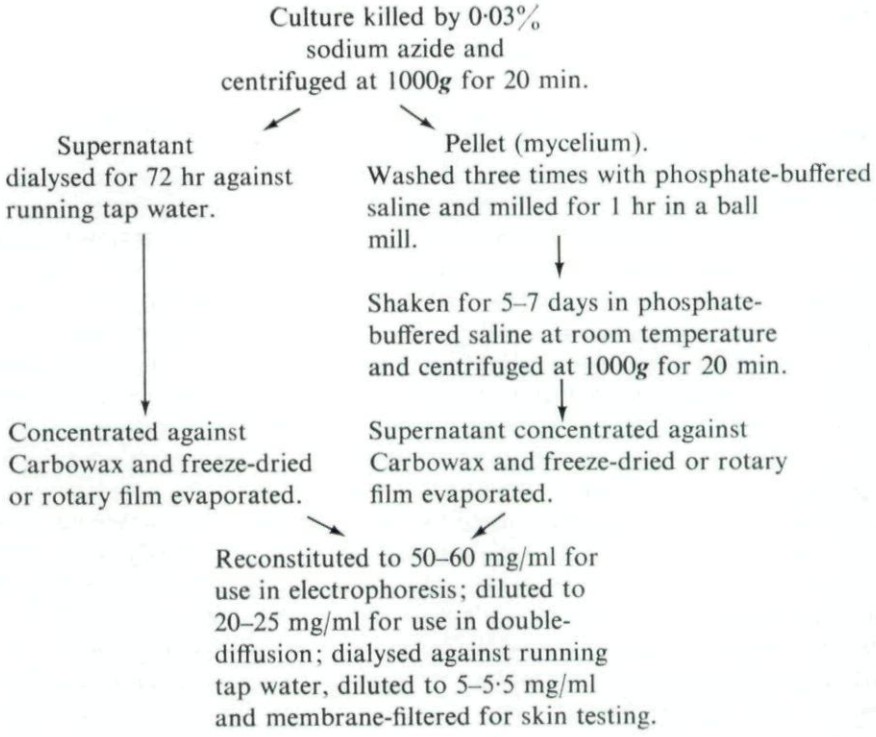

Fig. 2. Flow chart of antigen extraction.

phenolated glycerol at 5 mg per ml, were used as tests for occupational allergens Phenolated glycerol was used as a control.

Serum was separated from each of 711 20 ml samples of blood by centrifugation (1000*g* for 20 min) after overnight refrigeration at 4°C. All sera were made up to 0·03% or sodium azide and stored at −20°C before testing.

Double-diffusion (DD) was carried out in McIlvain's citrated agar (15 g Oxoid purified agar, 180 ml 0·1 M citric acid, 820 ml 0·2 M disodium hydrogen phosphate and 2·0 g sodium azide per l). Each serum was tested in a central well of 12·5 mm diameter surrounded by six peripheral 4 mm wells, cut by a template, in a 3 mm depth of agar in a 5·0 cm disposable Petri dish.

Immunoelectrophoresis (IE) and immunoelectro-osmophoresis (IEO) were conducted in 1·5% veronal agar, pH 8·2 (15 g Oxoid purified agar, 15·85 g sodium barbital, 230 ml 0·1 N hydrochloric acid, 770 ml distilled water per l). For IE, a pattern of antigen wells and antiserum troughs was cut as suggested by Pepys & Jenkins (1965); IEO was carried out according to the method of Gordon *et al.* (1971).

All reactions were allowed to take place over a period of 7 days in humid containers held at 26°C. Petri dish DD plates were soaked in five successive daily changes of isotonic saline, and were read wet in a viewing box after staining with 1% tannic acid (Alpert, Monroe & Schur, 1970). IE and IEO plates were washed in isotonic saline for 48 hr, dried under filter papers at 37°C, and arcs of precipitation were then stained with 1% Ponceau red, or 0·1% Amido black in 1·0 M acetic acid containing 0·1 M sodium acetate.

Results

Environmental cultures

The information given in Table 1, where organisms are arranged in descending order of overall environmental incidence, shows that *Penicillium* spp. were present in 80% of all samples whilst *Rhizopus stolonifer* and yeasts were subdominant. Eight species of *Penicillium* were identified as contaminants of maltings: *P. chrysogenum*, *P. cyclopium*, *P. citrinum*, *P. frequentans*, *P. granulatum*, *P. purpurogenum*, *P. piceum* and *P. viridicatum*, of which the first two species were the most common, with incidences of 60% and 50% respectively for all samples.

During open-floor malting, there was a characteristic increase in the incidences of fungi already present in or on the intake grain. *Alternaria* spp and *Epicoccum nigrum* remained comparatively constant, however, and a sharp decline in the number of samples yielding *Cladosporium* spp. (*C. herbarum* and *C. cladosporioides*) was notable. *Penicillium* spp. were dominant on the malt and *R. stolonifer* and yeasts were subdominant. Species of *Candida* and *Cryptococcus* together accounted for 74% of all environmental yeasts: *Rhodotorula* spp. were recorded in 20% and *Sporobolomyces roseus* in 9% of samples. The proportional representation of fungal species in the air above the malting floor was generally similar to that on the malt itself. The yeasts (*Cladosporium* spp., *Aureobasidium pullulans* and *Aspergillus* spp.) were more prevalent in the air, however.

Considering the results for Saladin box and other malting processes taken together, it can be seen that dominance relationships on the malt differed from those recorded on the open-floor material. The yeasts became dominant; the *Penicillium* spp. and *R. stolonifer* were subdominant and were closely followed by *Geotrichum candidum*. An unusually high incidence of 25% was recorded for *Fusarium* spp. *Aspergillus* spp., taken as a whole, reached a highest incidence in culms (25%) and dusts (15%), during the processing of which personnel are exposed to high concentrations of particles. Both these types of sample were rich in a wide range of fungal species.

24% of all environmental samples yielded *Aspergillus clavatus*; *A. fumigatus* occurred in 9% of samples, whilst *A flavus*, *A glaucus* and *A. niger* did not exceed incidences of 3%. A total of twelve maltings (21%) provided samples from which *A. clavatus* was cultured (Table 2), culms being positive in nine, dusts in six, intake grain in four and malt in three maltings. In only one malting was the fungus recovered from as many as four types of environmental sample.

Other fungi identified during the survey (Table 1) rarely exceeded 2% incidences, and included *Absidia* spp., *Acremoniella atra*, *Botrytis cinerea*, *Cephalosporium* spp., *Chaetomium globosum*, *Phoma* spp., *Piptocephalis* spp., *Torula herbarum*, *Trichothecium roseum*, *Verticillium* spp. and *Mycelia sterilia*.

Sputum smears and cultures

As is shown in Table 3 and Fig. 3, it was possible to identify higher plant cells, fungal mycelia and the spores of *Alternaria*, *Cladosporium*, the *Mucorales* and of *Penicillium* and/or *Aspergillus* in stained sputum smears. Budding blastospores and pseudomycelia of yeasts were also recorded. The percentage incidence of sputa positive for one or more of these elements was 70, and that for mycelia alone was 42. The type of sputum determined the yield, 78% of mucoid/mucopurulent sputa showing contaminants as opposed to 50% of watery sputa. Spores of *Penicillium* and/or

Table 1. Incidence of fungi in sputa and environmental samples

	Types and numbers of positive samples							
		Environment						
Fungi	Sputum [699]*	Intake grain [67]	Open floor malt [47]	Saladin or other malt [28]	Culms [56]	Dusts [73]	Air [56]	All samples [327]
Penicillium spp.	630	50	41	13	51	62	41	258
	(90)†	(75)	(87)	(46)	(91)	(85)	(73)	(79)
Rhizopus stolonifer	336	25	26	5	42	35	22	155
	(48)	(38)	(55)	(2)	(75)	(48)	(39)	(47)
Yeasts	369	13	22	15	23	25	38	136
	(53)	(19)	(47)	(54)	(41)	(34)	(68)	(42)
Alternaria spp.	34	38	14	11	4	13	14	94
	(5)	(57)	(30)	(39)	(7)	(18)	(25)	(29)
Cladosporium spp.	123	19	4	1	11	20	24	79
	(18)	(28)	(8)	(3)	(20)	(27)	(43)	(24)
Aureobasidium pullulans	33	15	2	4	8	19	10	58
	(5)	(22)	(4)	(14)	(14)	(26)	(18)	(18)
Aspergillus spp.	77	5	2	1	14	11	6	39
	(11)	(7)	(4)	(3)	(25)	(15)	(11)	(12)
A. clavatus	57	4	2	1	8	7	2	24
	(8)	(6)	(4)	(3)	(14)	(10)	(4)	(7)
A. fumigatus	4	1	0	0	3	1	4	9
	(1)	(1)	(0)	(0)	(5)	(1)	(7)	(3)
A. flavus	8	0	0	0	1	2	0	3
	(1)	(0)	(0)	(0)	(2)	(3)	(0)	(1)
A. glaucus	7	0	0	0	1	1	0	2
	(1)	(0)	(0)	(0)	(2)	(1)	(0)	(1)
A. niger	1	0	0	0	1	0	0	2
	(0·1)	(0)	(0)	(0)	(2)	(0)	(0)	(1)
Mucor spp.	60	1	9	5	7	11	3	34
	(9)	(1)	(19)	(18)	(12)	(15)	(4)	(10)
Geotrichum candidum	8	6	6	10	6	3	2	33
	(1)	(9)	(13)	(36)	(11)	(4)	(4)	(10)
Epicoccum nigrum	1	13	7	5	0	0	4	29
	(0·1)	(19)	(15)	(18)	(0)	(0)	(7)	(9)
Fusarium spp.	7	2	5	7	1	1	0	16
	(1)	(3)	(11)	(25)	(2)	(1)	(0)	(5)
Trichoderma viride	17	5	3	0	0	2	0	10
	(2)	(7)	(6)	(0)	(0)	(3)	(0)	(3)
Others	38	9	5	0	5	9	11	39
	(5)	(13)	(11)	(0)	(9)	(12)	(20)	(12)

* Totals in brackets. † Percentages in parentheses.

Table 2. Environmental and sputum sources of *A. clavatus* in relation to the occurrence of precipitins

Sources of *A. clavatus*	Maltings																	Totals
	A (54)*	B (12)	K (18)	O (9)	P (7)	P2 (20)	H (12)	C (7)	D (3)	B2 (8)	E (34)	K2 (28)	A2 (16)	H (3)	I (12)	I2 (7)		(250)
Intake grain		+						+		+	+							4
Open floor malt									+	+		+						3
Saladin and other malt							+											1
Culms	+				+	+	+	+	+	+	+	+						9
Dusts			+	+		+		+	+		+							6
Air										+		+						2
Number of men sputum-positive	1	2	1	0	7	0	0	5	0	6	4	25	1	2	2	1		57
Number of men precipitin-positive	6	3	0	0	6	0	1	5	0	8	20	26	0	1	0	0		76 (30%)

* Numbers in parentheses show total number of men in each malting.

Table 3. Occurrence of higher plant cells and fungi in sputum smears

	Materials identified							
				Spores				Total
	Higher plant cells	Mycelium	Yeasts	*Penicillium* and/or *Aspergillus*	Mucorales	*Cladosporium*	*Alternaria*	
Number of positive sputa	79 (14)*	239 (42)	62 (11)	71 (12)	70 (12)	32 (6)	8 (1)	401 (70)

* Percentages in parentheses.

Aspergillus, seen in 12% of smears, were a little more common than the blastospores of yeasts; spores of *Alternaria* and *Cladosporium* were each identified in less than 6% of all samples. Transient fungal contaminants were, therefore, a feature of the sputa of a significantly high number of men engaged in some aspect of malting. Mycelia were usually seen as fragments, or clumps, showing evidence of transverse fracture.

Table 1 shows the results of the culture of 699 sputa, of which 90% yielded *Penicillium* spp. *P. chrysogenum* was seen in over 70% of all cultures and *P. cyclopium* in 53%. Apart from a 19% incidence for *P. purpurogenum*, only *P. piceum* at 5% occurred as more than a trace. The *Mucorales* as a whole were seen in 59% of samples, and *R. stolonifer* appeared in 48%. 17% of sputa yielded *Cladosporium* spp. Yeasts of both exogenous and endogenous origin were identified in 53% of samples. 95% of sputum yeasts were *Candida* spp., 3% *Rhodotorula* spp. and 2% *Sporobolomyces roseus*.

Aspergilli were recovered from 11% of all sputa (Table 1), and the 8% which contained *A. clavatus* were from men working in eleven maltings, four of which gave no evidence of the fungus in environmental samples (Table 2).

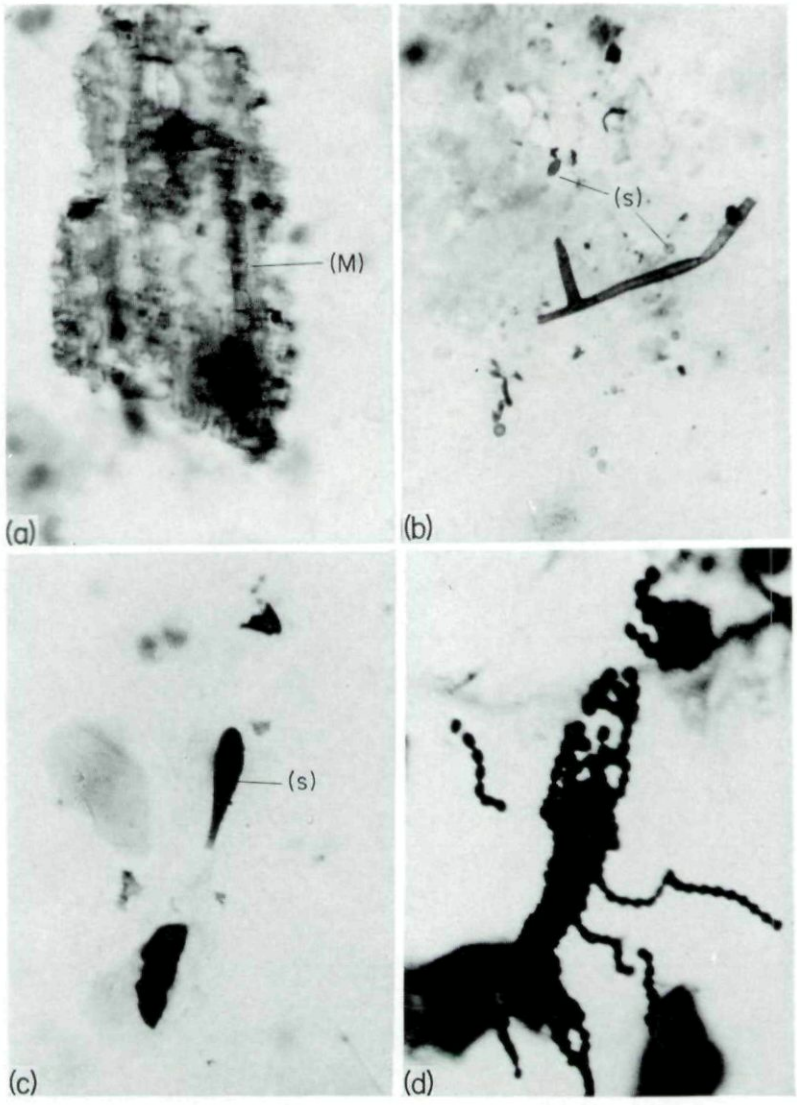

Fig. 3. Sputum smears stained by the methenamine-silver method. (a) Higher plant cells with associated fungal mycelium (M) (magnification ×175). (b) Septate fungal mycelium and the spores of *Cladosporium* (s) (magnification ×175). (c) a spore of *Alternaria* (s) (magnification ×840). (d) chains of spores of *Penicillium* or *Aspergillus* (magnification ×1050).

The dominant fungi of the environment were generally equally dominant in sputum. Some genera, notably *Aureobasidium*, *Alternaria*, *Epicoccum* and *Geotrichum*, only rarely appeared in sputum cultures, but were comparatively plentiful in the environment.

Immunology

Skin tests. Personnel giving a positive immediate reaction, as indicated by the development of a weal of at least 3 mm to one or more common allergens in prick

tests, were defined as atopic. There were 209 (29%) of whom 4·3% were positive for occupational fungi; 10·7% of non-atopics also reacted to these. Positive reactions to occupational allergens were recorded in 9% of all maltworkers and in 24% of those showing symptoms of allergic alveolitis.

Precipitins to fungal antigens. Sera from all 711 maltworkers, and fifty control sera from the Blood Transfusion Service, were tested by DD against mycelial (M) extracts from *Aspergillus clavatus*, *A. fumigatus*, *A. niger*, *Cladosporium herbarum*, *Rhizopus stolonifer* and *Trichoderma viride*, and against a culture filtrate (CF) extract from *Micropolyspora faeni* known to react with a large proportion of sera from cases of farmer's lung. No precipitation was recorded in control sera, and as is shown in Table

Table 4. Seropositivity of maltworkers to fungal mycelial antigens

	Number precipitin-positive to antigens				
	Aspergillus clavatus	*Aspergillus fumigatus*	*Aspergillus niger*	*Rhizopus stolonifer*	*Cladosporium herbarum*
	142 (20)*	157 (22)	73 (10)	24 (3)	117 (16)

	Cross-reactivity of the antigens				
	Aspergillus clavatus	*Aspergillus fumigatus*	*Aspergillus niger*	*Rhizopus stolonifer*	*Cladosporium herbarum*
A. clavatus	—	13 (9)	10 (7)	2 (1)	7 (5)
A. fumigatus	—	—	11 (7)	3 (2)	48 (31)
A. niger	—	—	—	10 (14)	35 (48)
R. stolonifer	—	—	—	—	11 (46)

* Numbers in parentheses show percentages (total = 711).

4, positive reactions were recorded for maltworkers' sera to all fungal extracts except those from *T. viride*; whilst a single positive reaction was recorded for *M. faeni*. The highest percentage sensitization was to *A. fumigatus* (22%), and in descending order to: *A. clavatus* (20%), *C. herbarum* (16%), *A niger* (10%) and *R. stolonifer* (3%). Of a total of 132 sera subsequently tested against an M extract from *Penicillium cyclopium*, nine (7%) gave arcs of precipitation. It was clear from the results, therefore, that a significant number of maltworkers was sensitized to weakly antigenic components from the mycelia of a range of commonly encountered environmental fungi, with which they were in daily contact. There was a high degree of cross-reactivity between extracts from *C. herbarum* and those from the other test fungi, with the exception of *A. clavatus*, implying the occurrence of antigenic components in common. None of forty sera tested against M extracts from *Alternaria tenuis*, *Aureobasidium pullulans*, *Geotrichum*

candidum, *Rhodotorula glutinis* or *Candida albicans* showed the presence of precipitating antibody.

A. clavatus in relation to disease

During the survey, 127 men were suspected of having symptoms suggestive of extrinsic allergic alveolitis (EAA), and were further investigated by respiratory physicians. Following examinations, each was allocated to one of five grades (0 to 4), comprising an index of EAA (Grant *et al.*, 1976). A score of 4 meant that characteristic symptoms of the disease had been identified, namely: cough, chest tightness and loss of breath, with or without fever occurring some hours after exposure to dust, and all symptoms disappearing during absence from work. A score of 0 was given to men who proved to be asymptomatic, or were suffering from some other form of respiratory disease. The intermediate grades of 1, 2 and 3 were allocated to men showing increasing numbers of significant symptoms, but not all of them. A score of 3 or 4 meant that disease was probable or certain, and 37 men in these categories made up 5·2% of all maltworkers surveyed.

Evidence that antigens from *A. clavatus* were significantly implicated in the disease came from the results on the environmental and sputum incidence of the fungus, in relation to the occurrence of serum precipitins (Table 2). Seventy-six (30%) of the 250 men from maltings where *A. clavatus* had been identified were precipitin-positive, compared to 14% of workers not directly known to have been in contact with the fungus; the two groups had incidences for EAA of 8% and 4% respectively.

Table 5 shows that seropositivity to *A. clavatus* M extracts increased from 12% in asymptomatic workers to 82% in workers with EAA grade 4. The highest numbers of reactions to all test fungi other than *A. clavatus* were recorded for grade 0 sera from individuals free of the disease. Reactions in other EAA grades were few, and of no significance in diagnosis. CF extracts from two separate isolates of *A. clavatus* were tested by DD against the 127 sera from suspected cases, and against another twenty-six randomly selected from the remaining workers. Of the only five sera to give precipitation reactions, two were grade 0, one was grade 2 and two were grade 4. All 711 sera from maltworkers, and the fifty control sera, were tested to one CF extract by IEO. Thirty-seven maltworkers were seropositive (Table 5). It appeared that CF antigens were ineffective as an aid to diagnosis and, compared to M antigens, were of low immunogenicity.

Immunoelectrophoresis (IE), using a composite developing serum derived from aliquots of four highly-reactive grade 4 sera, showed that two main groups of antigens (2 and 3) could be identified in M extracts and three (1, 2 and 3) in CF extracts from *A. clavatus* (Fig. 4). A positively charged antigen of group 1 migrated towards the cathode and was only recorded in CF extracts. The weakly charged group 2 remained near the origin, that from M extracts comprising four antigens and that from CF extracts, two. A single negatively charged group 3 antigen occurred in culture filtrates, but four antigens were identified in M extracts.

Discussion

Barley grain at harvest is known to possess a fungal flora characteristically dominated by genera such as *Alternaria*, *Cladosporium*, *Fusarium* and *Mucor*, which are referred to as field fungi (Mulinge & Chesters, 1970). Flannigan (1969) has shown that after about 1 month, fungi such as *Penicillium* spp. (members of the storage fungi) are

Table 5. Seropositivity to fungal extracts in relation to diagnostic grade of EAA

	Number with positive reactions					
	No clinical assessment	EAA diagnostic grade				
Extract	[584]*	0 [74]	1 [3]	2 [13]	3 [15]	4 [22]
Aspergillus clavatus (M)	69(12)†	13(18)	1(33)	7(53)	9(60)	18(82)
Aspergillus clavatus (CF)	0‡	2	0	1	0	2
Aspergillus clavatus (CF) IEO	29	6	0	0	0	2
Aspergillus fumigatus (M)	141	16	0	0	0	0
Aspergillus niger (M)	65	7	0	1	0	0
Aspergillus flavus (M)	n.t.	n.t.	n.t.	0**	1††	2‡‡
Aspergillus terreus (M)	n.t.	n.t.	n.t.	0**	0††	1‡‡
Cladosporium herbarum (M)	110	6	0	0	1	0
Rhizopus stolonifer (M)	22	1	0	0	0	1
Penicillium cyclopium (M)	8§	0¶	0	0**	0††	1‡‡
Micropolyspora faeni (CF)	1	0	0	0	0	0
Trichoderma viride (M)	0	0	0	0	0	0
Alternaria tenuis (M)	n.t.	n.t.	n.t.	0**	0††	0‡‡
Aureobasidium pullulans (M)	n.t.	n.t.	n.t.	0**	0††	0‡‡
Candida albicans (M)	n.t.	n.t.	n.t.	0**	0††	0‡‡
Rhodotorula glutinis (M)	n.t.	n.t.	n.t.	0**	0††	0‡‡

n.t. = Not tested.

* Numbers in brackets show totals. † Numbers in parentheses show percentages. ‡ Out of twenty-six. § Out of eighty-six. ¶ Out of six. ** Out of eight. †† Out of eleven. ‡‡ Out of twenty-one.

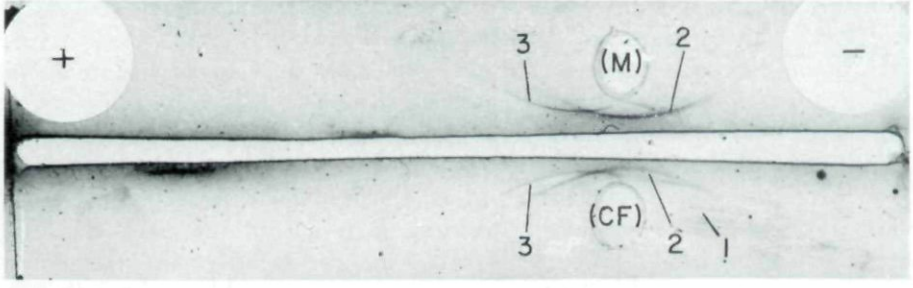

Fig. 4. Immunoelectrophoresis of mycelial (M) and culture filtrate (CF) extracts of *A. clavatus* with composite serum from maltworkers. Three groups of antigens are shown.

already present in some concentration, and by 2 to 3 months become dominant. It is assumed that intake grain samples cultured during the present survey had been stored for about this time, as *Aspergillus* spp., usually encountered after longer periods of storage, were still comparatively rare. The dominance relationships in fungal groups during malting were determined largely by the type of malting process employed. Yeasts were dominant in the Saladin box and enclosed systems, and species of *Penicillium*, *Alternaria* and *Geotrichum* were subdominant; whereas on open floors the

Penicillium spp. were clearly dominant and the yeasts, together with *Rhizopus stolonifer*, were subdominant. Recovery of *Aspergillus clavatus* was about the same (4%) in samples of malt from open floors and those from all the other systems taken together. The occurrence of respiratory disease and immunological features associated with proximity to vegetable products subject to biodeterioration have been reviewed by Pepys (1969), who has emphasized that large numbers of organic materials may provoke antibody production after inhalation. Riddle (1974), in a survey of 114 maltworkers, showed that 68 (60%) had precipitins to antigens from one, or several, of four environmental fungi, 10% being to those from *A. clavatus*. Although not all maltworkers' sera in the present work were tested against extracts of the various test fungi used, a total of 409 (57%) was positive to one or more of them. In view of the absence of such antibodies in control sera, their role must remain obscure, as no correlation between their occurrence and the presence of disease has been established, except for those induced by antigens from *A. clavatus*. It is of interest that only six out of the selected twelve test fungi were precipitinogenic.

The allergenic fungus, *A. clavatus*, apparently has a low incidence in the air and in malt from both open-floor and other systems when the industry is viewed as a whole. Of the twelve maltings with positive environments, six showed contamination of only one type of sample, and of the 120 men comprising the workforce, precipitins to *A. clavatus* were detected in fifteen (12·5%). In the remaining six maltings, five of which yielded multiple sources of contamination, sixty men (65%) were precipitin-positive out of ninety-two. In what were assumed to have been minimally contaminated maltings, where no environmental samples were positive and sputa were the only sources of allergen, only one man (2·6%) had precipitins out of 38 (Table 2). The concentration of fungal propagules apparently directly determined the incidence of sensitization, and the 250 men from these maltings had the highest recorded occurrence of allergic alveolitis of any group in the survey (7·6%). Grant *et al.* (1976) commented on the fact that as 24% of men showing symptoms of extrinsic allergic alveolitis also had an immediate skin sensitivity to fungal occupational antigens, in comparison to only 9% of all maltworkers, the disease may also incorporate a type I hypersensitivity component. Ghose *et al.* (1974) have presented some evidence for the triggering of farmer's lung by a type I hypersensitivity.

It has been established that the overall sputum culture incidence of the fungus was 8%. As there were no means available for the specific identification of the organism in sputum smears, it is not known whether it was present in the form of spores, mycelial fragments or both. The significance of M antigens in sensitization and disease has, however, been amply demonstrated, and whether they are surface-bound wall components, as well as being intracellular components, is not known. The fact that broken, unidentifiable mycelial fragments were found in no fewer than 42% of all smears would suggest that intracellular fungal antigens are equally freely in contact with the respiratory tract as the surface components. It is known that after a single exposure of volunteer personel, not associated with the industry, during the turning of malt colonized by *A. clavatus*, the fungus was subsequently detectable in sputum cultures for 4 weeks (Channell *et al.*, 1969). Clearance by the mucociliary escalator may, therefore, be a comparatively slow process, and exposure to antigens could be of considerable duration.

The lack of cross-reactions between antibodies to *A. clavatus* and antigens from other fungi is of some significance, particularly in relation to sera from men in

the EAA diagnostic grades. In all grades other than 0, reactions to antigens from other fungi were almost completely absent (Table 5), thus emphasizing the diagnostic value of M antigens of *A. clavatus* in the serological confirmation of clinical evaluations. The prevalence of antibodies to *A. fumigatus* was predictable, as they are known to be widely distributed in sera from both healthy donors and those showing various manifestations of aspergillosis (Bardana, 1974).

The highest percentage occurrence of *A. clavatus* in environmental samples was recorded for dusts (10%) and culms (14%). Riddle (1974) mentioned six men from his survey who stated that they were unable to smoke during the evening after cleaning culms out of bins. It is probable that such work involved the inhalation of large amounts of allergenic dust. As is shown in Fig. 1, dusts from screening and culms may also be incorporated into cattle feed. *A. clavatus* is known to synthesize patulin (Florey, Jennings & Philpot, 1944; Katzman *et al.*, 1944) and cytochalasin E (Glinsukon *et al.*, 1974). The fungus is recognized as the cause of haemorrhagic syndrome in poultry and of hyperkeratosis and hepatic degeneration in calves (Forgacs & Carll, 1962; Moreau & Moreau, 1960). Experimental granulomatous and mycotoxic syndromes have been described in mice by Blyth & Lloyd (1971), who also isolated a mycelial tremorgen by acid hydrolysis. Glinsukon *et al.* (1974), using silica gel chromatography, extracted two tremorgens from an isolate of *A. clavatus* associated with a case of fatal human disease in Thailand. They were identified as tryptoquivaline and tryptoquivalone by Clardy *et al.* (1975). The role of the fungus in the provocation of respiratory disease and mycotoxicosis is now fully appreciated. The avoidance of high ambient malting temperatures conducive to enhanced microbial growth (Riddle *et al.*, 1968), and the judicious use of face masks or respirators when high concentrations of fungal contaminants are encountered are clearly indicated.

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References

Alpert, E., Monroe, M. & Schur, P.H. (1970) A method for increasing the sensitivity of radial immunodiffusion assay. *Lancet*, **i,** 1120.

Bardana, E.J. (1974) Measurement of humoral antibodies to Aspergilli. *Annals of the New York Academy of Sciences*, **221,** 64.

Blyth, W. & Lloyd, M.M. (1971) Granulomatous and mycotoxic syndromes in mice due to *Aspergillus clavatus* Desm. *Sabouraudia*, **9,** 263.

Channell, S., Blyth, W., Weir, D.M., Lloyd, M.M., Amos, W.M.G. & Grant, I.W.B. (1969) Allergic alveolitis in maltworkers: a clinical, mycological and immunological study. *Quarterly Journal of Medicine*, **38,** 351.

Clardy, J., Springer, J.P., Büchi, G., Matsuo, K. & Wightman, R. (1975) Tryptoquivaline and tryptoquivalone, two tremorgenic metabolites of *Aspergillus clavatus*. *Journal of the American Chemical Society*, **97,** 663.

Flannigan, B. (1969) Microflora of dried barley grain. *Transactions of the British Mycological Society*, **53,** 371.

Florey, H.W., Jennings, M.A. & Philpot, F.J. (1944) Claviformin from *Aspergillus giganteus* (Wehm.). *Nature (London)*, **153,** 139.

Forgacs, J. & Carll, W.T. (1962) Mycotoxicoses. *Advances in Veterinary Science*, **7,** 273.

Ghose, T., Landrigan, P., Killeen, R. & Dill, J. (1974) Immunopathological studies in patients with farmer's lung. *Clinical Allergy*, **4,** 119.

GLINSUKON, T., YUAN, S.S., WIGHTMAN, R., KITAURA, Y. BÜCHI, G., SHANK, R.C., WOGAN, G.N. & CHRISTENSEN, C.M. (1974) Isolation and purification of cytochalasin E and two tremorgens from *Aspergillus clavatus*. *Plant Foods for Man*, **1,** 113.

GORDON, M.A., ALMY, B.E., GREENE, C. & SENTON, J.W. (1971) Diagnostic mycoserology by immunoelectroosmophoresis: a general, rapid and sensitive microtechnique. *American Journal of Clinical Pathology*, **56,** 471.

GRANT, I.W.B., BLACKADDER, E.S., GREENBERG, M. & BLYTH, W. (1976) Extrinsic allergic alveolitis in Scottish maltworkers. *British Medical Journal*, **1,** 490.

GROCOTT, R.G. (1955) Stain for fungi in tissue sections and smears using Gomori's methanamine silver technique. *American Journal of Clinical Pathology*, **25,** 975.

KATZMAN, P.A., HAYS, E.E., CAIN, C.K., WYK, J.J. VAN, REITHEL, F.J., THAYER, S.A. & DAISY, E.A. (1944) Clavacin, an antibiotic substance from *Aspergillus clavatus*. *Journal of Biological Chemistry*, **154,** 475.

MOREAU, C. & MOREAU, M. (1960) Un danger pour le bétail nourri de plantules fourrangeres cultivées en germoirs: la population d'une moisissure toxique, *l'Aspergillus clavatus*, cause de accidents mortels. *Comptes rendues hebdomadaire des seances de l'Academie d'Agriculture de France*, **46,** 441.

MULINGE, S.K. & CHESTERS, C.G.C. (1970) Ecology of fungi associated with moist stored barley. *Annals of Applied Biology*, **65,** 277.

PEPYS, J. (1969) *Hypersensitivity Diseases of the Lungs due to Fungi and Organic Dusts*. Kager, Basel and New York.

PEPYS, J. & JENKINS, P.A. (1965) Precipitin (FLH) test in farmer's lung. *Thorax*, **20,** 21.

RIDDLE, H.F.V. (1974) Prevalence of respiratory symptoms and sensitization by mould antigens among a group of maltworkers. *British Journal of Industrial Medicine*, **31,** 31.

RIDDLE, H.F.V., CHANNELL, S., BLYTH, W., WEIR, D.M., LLOYD, M.M., AMOS, W.M.G. & GRANT, I.W.B. (1968) Allergic alveolitis in a maltworker. *Thorax*, **23,** 271.