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Experimental aspergillosis in mice: aspects of resistance

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SUMMARY

Intravenous inoculation of *Aspergillus fumigatus* spores was used to study experimentally induced and natural resistance. Slight resistance resulted in increased survival time and higher resistance produced in addition a decreased infection rate.

Sublethal doses of living spores gave significant protection against challenge 3 weeks later, but large doses of heat-killed spores had no demonstrable effect.

Mice from one source showed a single, dramatic decrease in dose response to a deep-frozen strain of the organism over a period of 34 months. The dose response initially resembled that described by Scholer (1959) in which one million spores killed the majority of mice. The change was almost certainly due to an increase in resistance of the mice due to environmental factors, and the resistance was probably also effective against other strains of the organism. Although not proved, it seemed likely that the resistance was due to increased natural contact with *A. fumigatus* or related fungi. Possibly for a similar reason, mice of the same stock bred on different premises differed in their susceptibility to infection. The results indicated that environmental resistance-producing factors may have been operating simultaneously on a number of premises housing laboratory animals in south-east England. These findings may have significance in relation to the occurrence of natural aspergillosis of mammals and birds.

Of five *A. fumigatus* strains, four were of closely similar virulence; the fifth strain grew more slowly *in vitro* and was somewhat less virulent. Isolates from mice which died sporadically after small doses of spores were of no greater virulence than the inoculated strain. Although the susceptibility of mice aged 3 weeks was not uniform under all conditions, such animals were less resistant than young adult mice. Mice from six different sources showed only slight differences in susceptibility between each other, or from mice known to have developed a natural resistance.

INTRODUCTION

Vaccination against mycoses is almost untried (Smith, 1969) and experiments on immunization (Kong & Levine, 1967) have usually been concerned with fungi whose growth *in vivo* is characterized by multiplication of individual organisms.

Aspergillus fumigatus, although basically saprophytic, is an important pathogen of birds and mammals. When growth occurs in the depths of animal tissues, it

does so without increase in the number of organisms. Human allergic disease and the demonstration of antibodies *in vitro* (Austwick, 1965; Pepys, 1969) have attracted considerable study, but precise information on increased resistance to infection as a result of previous contact with the organism or its products is scanty and often inconclusive.

Henrici (1939) considered that hyperimmunization with endotoxin immunized four out of six rabbits against inoculation of spores, but Tilden, Hatton, Freeman & Williamson (1957) and Tilden *et al.* (1961) reported that rabbits which had proved immune to large doses of toxin failed to resist the intravenous inoculation of 10^7 spores. They also found that neither a previous non-fatal infection nor inoculation of heated spores protected rabbits against fatal doses. Asakura, Nakagawa, Masui & Yasuda (1962) stated that resistance seemed to be conferred on a group of five ducks by subcutaneous inoculation of living spores: although all showed symptoms after intravenous challenge only one died, whereas three out of five control birds succumbed. Austwick (1962) reported that 66 % of slaughtered dairy cows showed minute pulmonary lesions termed 'asteroid bodies' and that *A. fumigatus* was isolated; similar bodies were found by King, Munday & Hartley (1965) who considered that the pathology indicated an immune response. O'Meara & Chute (1959) and Klimeš & Rosa (1964) found that chicks developed considerable resistance within the first few days of life to infection by inhalation.

Scholer (1959) studied aspergillosis induced in mice by intravenous inoculation of spores and found that, although slight variations in virulence occurred among six strains of *A. fumigatus*, most animals died within 20 days when given one million or more spores. Ford & Friedman (1967) reported that three strains of *A. fumigatus* were of similar virulence for mice by intravenous inoculation, but the dose response may have been somewhat lower than that described by Scholer (1959).

Although such infections are highly artificial, the ready availability of mice constitutes an important advantage in studies which demand large numbers of animals. This paper describes the use of mice inoculated intravenously with spores for the investigation of certain aspects of resistance to *A. fumigatus*.

MATERIALS AND METHODS

Purification and preservation of A. fumigatus *strains*

Cultures were received at the laboratory after storage at room temperature for 9–90 days. On receipt, the purity of each strain was ensured as follows. Serial dilutions of a nutrient broth suspension of well-separated spores were sampled on Sabouraud's dextrose agar (Oxoid, CM 41). After incubation at 37° C. for about 21 hr., a piece of agar bearing a single unsporulated colony was transferred to a fresh sterile container and incubated for a further 24 hr. The spores thus produced were suspended in saline and used to inoculate a large batch of Sabouraud's dextrose agar cultures which, after incubation for 3 days, was preserved at either −20° C. or 4° C.

Strains of A. fumigatus

Strain AF_1 was isolated from a black-footed penguin which died in the Zoological Society's Collection at Regent's Park from pulmonary aspergillosis. This strain was used exclusively for all experiments except one. The work was carried out during a period of 34 months and, for each experiment, the organism was obtained from a single batch of purified cultures stored at $-20°$ C.

Strains AF_2 and AF_3 were isolated from pulmonary aspergillosis in a citron crested cockatoo at Whipsnade and a Chilean flamingo in Gloucestershire; after purification, these strains were kept at 4° C. and used 1 month later. Strain AF_4 was isolated from pulmonary aspergillosis in a yellow-fronted Amazon parrot in Yorkshire and, after purification, was stored for 3 weeks at $-20°$ C. before use. Strain AF_5 was cultured from the pneumonic lung of a day-old Demidoff's bush-baby which died at Regent's Park, but its pathogenic significance was uncertain; it was purified and stored in the same way as strain AF_4.

Preparation of spore suspension for intravenous inoculation

Sabouraud's dextrose agar was poured into horizontal medical flat bottles. The bottles were inoculated with spore suspension and incubated at 37° C. for 3 days. Nutrient broth (Oxoid, CM 67) was then introduced through the rubber liners of perforated bottle caps by means of a hypodermic syringe and needle. After thorough agitation, the bottles were inverted and spore suspension was withdrawn by syringe from the centre of the fluid column, thus avoiding clumps of spores, the majority of which either rose to the surface or sedimented. After further thorough agitation, the spores were washed 3 times, resuspended in nutrient broth to the opacity of Brown's tube 19 and checked microscopically to ensure that the great majority were singly dispersed. Such a suspension could be expected to contain approximately 2×10^8 viable spores per ml., and dilutions in nutrient broth were prepared for the immediate intravenous inoculation of mice in 0·25 ml. volumes. Because spores sedimented rapidly, suspensions were quickly shaken on each occasion before filling a syringe. A retrospective check on viable count was always carried out, by sampling 0·1 ml. volumes of decimal dilutions on Sabouraud's dextrose agar plates and counting colonies after 21 hr. incubation; the mean of three replicate counts was taken as the true value.

Heat-killed spore suspension

A 100 ml. medical flat bottle containing 30 ml. of a nutrient broth spore suspension ($24·8 \times 10^6$ viable spores per ml.) was immersed for 15 min. in a water bath at 65° C. and then immediately cooled. Sterility was checked by culturing 0·3 ml. in Sabouraud's liquid medium (Oxoid, CM 147).

Mice

Unless stated otherwise, the mice consisted of females weighing approximately 20 g., purchased from supplier A ('SA' mice). Mice designated SB, SC, SD, SE_1, SE_2 and SF were purchased from five further suppliers. All animals were Swiss

white mice from outbred, closed colonies and they consisted of different strains with the exception of SD and SE_1 mice, which represented two different lines of the same strain. The SE_2 and SF mice were specific pathogen free, but they were not barrier-maintained at the Institute. Mice were used within a week of arrival, except where stated otherwise. At the Institute, mice were fed a pelleted form of diet 86 (Howie, 1952), obtained throughout from a single manufacturer.

Cultural examination of mice

In initial studies on the nature of the infection, a loopful of brain tissue and the cut surfaces of lung, liver, spleen and kidney were smeared on Sabouraud's dextrose agar and incubated for 2 days at 37° C. In later studies, renal tissue was examined by culturing any obvious lesion and a large loopful of homogenized suspension of both kidneys. Carcasses were frequently stored at −20° C. before examination.

RESULTS

Experimental aspergillosis in mice

The pathology of the disease which follows intravenous inoculation of *A. fumigatus* spores has already been described by Scholer (1959) and additional observations only are given here. Detailed information on the effects of various doses on mortality is presented later.

Lung, liver, spleen, kidney and brain from mice which died in 1 or 2 days as a result of large doses of spores were almost invariably positive on culture. Multiple, minute, whitish foci were visible in both kidneys of animals dying after the second or third day, and these became progressively larger as the time to death increased; the lesions were very obvious in mice dying after about 7 days. As judged by culture, infection was progressively lost, first from the lungs, then spleen and liver, and somewhat later from brain, so that in animals dying 10 or more days after inoculation, infection was almost invariably confined to the kidneys. Mice dying after several weeks usually showed large lesions in both kidneys, and survivors slaughtered after prolonged periods were usually either normal at post-mortem or severely affected in one kidney only. A week or more after inoculation, there was very close correlation between the presence or absence of macroscopic kidney lesions and positive or negative cultures.

Experimental production of resistance

Two groups of 104 SA mice were inoculated intravenously with either 62,000 or 6200 living spores of strain AF_1. Two further large groups were similarly treated in dose volumes of 0·25 ml. with either $6{\cdot}2 \times 10^6$ heat-killed spores or sterile nutrient broth (controls). After 3 weeks, during which the larger dose of living spores produced nine deaths (8·6 %) and the smaller dose none, the four main groups were used to supply subgroups of 11–15 mice for intravenous challenge with doses of 60, 30, 6, 3, 0·6, 0·06 and 0·006 million spores. Deaths were recorded at least once a day, and all survivors were killed 64 days after challenge. To monitor the effects of the original doses of living spores, 13 and 20 mice treated respectively with

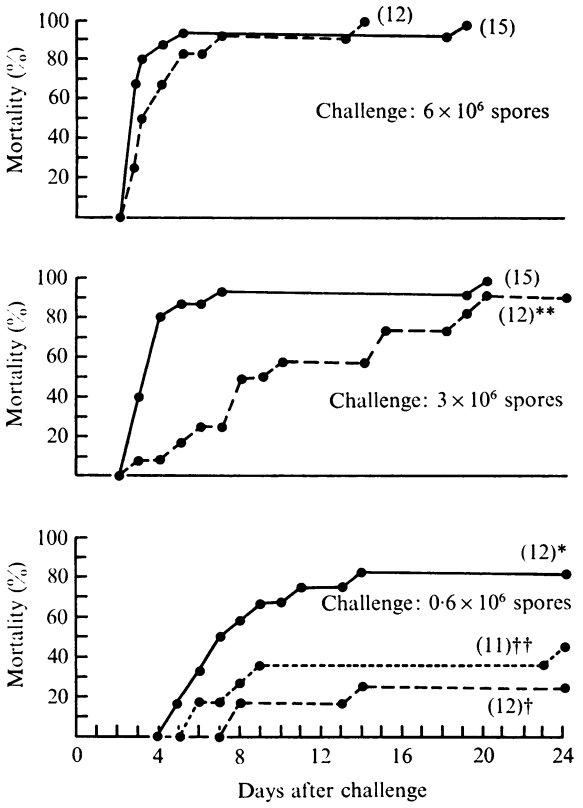

Fig. 1. Production of resistance to *A. fumigatus* by intravenous inoculation of sublethal doses of living spores. Mice treated with 62,000 spores (———), 6200 spores (- - - - -) and sterile nutrient broth (——) 3 weeks before intravenous challenge. Figures in parentheses indicate number of mice in group. ** = 100% mortality 64 days after challenge; * = 83% mortality 64 days after challenge; †† = 64% mortality 64 days after challenge; † = 50% mortality 64 days after challenge.

62,000 and 6200 spores were left unchallenged; the larger dose produced two deaths from renal aspergillosis and a further four infected animals were found at slaughter, but the smaller dose was without apparent effect.

Challenge doses of 60×10^6 and 30×10^6 spores were fatal to all mice within 5 days, while $0{\cdot}06 \times 10^6$ had killed only three of 12 controls, and $0{\cdot}006 \times 10^6$ none of 12 controls, by the end of the experiment. However, doses of an appropriate size showed clearly (Fig. 1) that an earlier sublethal infection produced resistance, manifested in this experiment as an increase in survival time. A dose of 6200 spores produced resistance to challenge with $0{\cdot}6 \times 10^6$ spores ($P < 0{\cdot}025$ on days 14–23), but the larger dose of 62,000 was more effective ($P < 0{\cdot}005$ on days 11 to 24). This larger dose also resulted in increased survival time of mice challenged with 3×10^6 spores ($P < 0{\cdot}0005$ on day 4 to $P < 0{\cdot}0125$ on day 9) and 6×10^6 spores ($P < 0{\cdot}04$ at 65 hr.), but the observable effect diminished as challenge increased. The dose of 6200 spores failed to protect against challenge of 3×10^6 and 6×10^6 spores. The 8·6% mortality which occurred before challenge in mice pretreated

Table 1. *Increased resistance of mice following inoculation of a sublethal dose of living spores*

	Total deaths at intervals (days) after challenge				
	5	10	14	28	69
Control mice	17/30	24/30	26/30	29/30	29/30
Pre-infected mice	3/12*	5/12	5/12	6/12	7/12
$P <$	NS	0·02	0·003	0·0005	0·002

Intravenous challenge of $0{\cdot}9 \times 10^6$ spores given 3 weeks after a pre-infecting dose of $0{\cdot}03 \times 10^6$ spores.

* Two of the deaths shown occurred before challenge; they were due to renal aspergillosis.

NS = not significant.

with 62,000 spores may have removed a small number of the more susceptible animals, but this could not have accounted for the considerable resistance produced. Moreover, any mortality occurring after challenge as a result of pretreatment with 62,000 spores would have biased the experiment against the demonstration of protection. When killed, the survivors of the groups depicted in Fig. 1 almost always showed a lesion in one kidney, from which *A. fumigatus* could be isolated. The narrowness of the range of challenge doses over which resistance could be demonstrated was probably a reflexion of the organism's inability to multiply *in vivo*. The experiment gave no indication that mice pretreated with heat-killed spores differed from the controls.

A further test was made with mice kept, in this instance, at the Nuffield Institute for 4 weeks between purchase and the commencement of the experiment. Twelve mice were pretreated with $0{\cdot}03 \times 10^6$ spores intravenously and 30 control mice were left untreated. The animals were challenged intravenously with $0{\cdot}9 \times 10^6$ spores after three weeks, during which period two test mice died from renal aspergillosis. Despite the mortality produced by the preinfecting dose, the test mice showed significantly lower mortality than the controls from the tenth day after challenge until the experiment was terminated on day 69 (Table 1). Thus, significant protection was of greater duration than in the first experiment. Of the five surviving test mice, four were found at slaughter to have *A. fumigatus* infection of one kidney.

Natural occurrence of resistance

The work described above formed part of a series of experiments on aspergillosis in mice carried out between April 1969 and January 1972. Six experiments performed during the period April 1969 to August 1970, each demonstrated a dose response similar to that reported by Scholer (1959) in that a dose of about 10^6 spores was rapidly fatal to most mice. The experiment shown in Table 1 was carried out at the end of August 1970, and is worthy of special mention as the control mice showed the usual susceptibility, even though they had in this instance been kept at the Institute for 7 weeks between purchase at 20 g. weight and inoculation. Again, the control mice shown in Fig. 1 were kept at the Institute for 4 weeks before challenge, yet $0{\cdot}6 \times 10^6$ spores produced 83% mortality in 14 days.

Table 2. *Spontaneous change in response of mice from a closed colony to* Aspergillus fumigatus *spores grown from a single batch of frozen cultures*

Reciprocal dilution of spores	Days after inoculation: 2 A*	2 B*	5 A	5 B	8 A	8 B	64 A	64 B
1	12	7	12	12	12	12	12	12
2	12	0	12	8	12	8	12	12
4	9	0	12	6	12	10	12	12
8	5	0	11	0	11	6	11 (1)	11 (1)
16	0	0	12	0	12	4	12	7 (4)
32	1	0	8	0	11	0	12	1 (5)
64	0	0	9	0	10	0	11 (0)	3 (1)
128	0	0	3	0	5	0	9 (2)	0 (7)
256	0	0	1	0	3	0	8 (3)	1 (2)
512	0	0	0	0	0	0	3 (6)	0 (0)
1,024	0	0	1	0	1	0	2 (7)	0 (1)
2,048	0	0	0	0	0	0	0 (3)	0 (1)
4,096	0	0	0	0	0	0	0 (2)	0 (1)
8,192	0	0	0	0	0	0	0 (2)	0 (0)
16,384	0	0	0	0	0	0	0 (0)	0 (0)

Figures indicate deaths in groups of 12 mice after inoculation with dilutions of spore suspension.

Figures in parentheses indicate number of survivors showing active *A. fumigatus* lesions at slaughter, 64 days after inoculation.

* Expt. A was begun in May 1970: the 1/32 dose contained $1{\cdot}43 \times 10^6$ viable spores. Expt. B was begun in May 1971: the 1/32 dose contained $1{\cdot}71 \times 10^6$ viable spores.

At some time between August and November 1970, the response of the SA mice to spores grown from the deep-frozen AF_1 cultures underwent a change, and from December 1970 to January 1972, each of 15 experiments showed a dose response which was greatly reduced and, though it fluctuated slightly on occasion, was on the whole remarkably constant. One effect was that 10^6 spores no longer rapidly killed the majority of mice.

The magnitude of the change is indicated in Table 2, which shows virulence titrations carried out in May 1970 (Expt. A) and May 1971 (Expt. B), under conditions as nearly identical as possible. The dramatic change in dose response was related to increased survival time and also to reduced infection rate, as was shown by examination of survivors killed 64 days after inoculation. The final LD 50 values (Reed & Muench, 1938) in Expts. A and B were $0{\cdot}146 \times 10^6$ and $2{\cdot}75 \times 10^6$ spores respectively, differing by a factor of approximately 18. The corresponding ID 50 values were $0{\cdot}034 \times 10^6$ and $0{\cdot}9 \times 10^6$, differing by a factor of approximately 26. These differences are impressive in view of the organism's inability to multiply *in vivo*.

Every effort was made to adhere throughout to standard technique in the preparation and inoculation of spore suspensions. Nevertheless, an examination of technique was made and the results, which were entirely negative, are summarized as follows. Spores grown on two different manufacturer's batches of

Table 3. *Virulence of five strains of* Aspergillus fumigatus *for SA mice*

Dilutions of Brown's no. 19 spore suspension	Deaths in groups of 12 mice after inoculation with dilutions of spore suspensions prepared from five strains					
	Expt. 1: results recorded 21 days after inoculation			Expt. 2: results recorded 28 days after inoculation		
	AF_1	AF_2	AF_3	AF_1	AF_4	AF_5
1/5	12	5 (5)	12	11 (1)	11 (1)	12
1/10	11 (1)	2 (9)	4 (7)	8 (3)	6 (6)	9 (3)
1/20	4 (8)	2 (2)	3 (7)	8 (3)	6 (3)	5 (4)
1/40	1 (5)	0 (4)	3 (4)	2 (7)	3 (4)	3 (5)
1/80	1 (7)	2 (4)	2 (8)	2 (6)	2 (3)	4 (4)
1/160	0 (4)	0 (2)	1 (2)	1 (5)	1 (2)	0 (4)

Figures in parentheses indicate survivors showing active *A. fumigatus* lesions at slaughter. The 1/40 doses contained the following numbers of viable spores (millions): AF_1 (Expt. 1), 1·46; AF_2, 1·15; AF_3, 1·05; AF_1 (Expt. 2), 1·32; AF_4, 1·39; AF_5, 1·47.

Sabouraud's dextrose agar, and on medium subjected to excessive autoclaving, did not differ significantly in their effects on mice. Slight differences in the age of cultures from which spores were obtained did not affect mortality. Spore suspensions made up independently by two individuals produced similar mortality.

Supplier A, a well-known and reputable breeder, gave a categorical assurance that he had not consciously changed the management, feeding or breeding of the SA closed colony.

The explanation of the events observed clearly lay either in a decrease in virulence of strain AF_1 or in an increase in the resistance of the mice due to environmental factors; increased resistance due to genetical factors appeared to be ruled out by the circumstances. The following experiments were designed to investigate the cause of the change in dose response and to give a background of information against which it could be viewed.

Comparison of the virulence for SA mice of five strains of A. fumigatus

Loss of virulence of strain AF_1 always seemed unlikely because the purified seed cultures were stored at $-20°$ C.; in addition, the change in dose response took place suddenly rather than gradually. Nevertheless, it was conceivable that slow progressive loss of viability at $-20°$ C. in a possibly heterogeneous population of spores might have resulted in selection leading to reduced virulence. This was examined in the light of Scholer's (1959) observation, based on examination of six strains, that 10^6 spores killed the majority of mice – a dose response which resembled that of the SA mice to strain AF_1 before the autumn of 1970. Accordingly, comparisons between AF_1 and four more recently isolated strains were made in the course of two experiments separated by an interval of 16 weeks (Table 3). It seemed reasonable to argue that if strain AF_1 had lost virulence during storage, it should be noticeably less virulent than at least some, if not all, of the four additional strains. Table 3 shows that this was not so.

Doses slightly greater than 10^6 spores produced consistently low mortality and

Table 4. *Virulence of strain AF_1 after one mouse passage*

Days after inoculation	Deaths in mice inoculated with doses of spores from: Original strain AF_1 $1{\cdot}1 \times 10^6$	six isolates of strain AF_1 after passage through mice: $1{\cdot}7 \times 10^6$	$1{\cdot}3 \times 10^6$	$1{\cdot}7 \times 10^6$	1×10^6	1×10^6	$0{\cdot}9 \times 10^6$
12	3/12	2/8	1/8	1/8	2/8	2/8	1/8
28	4/12	2/8	4/8	2/8	2/8	2/8	2/8

no significant differences in virulence emerged except that, in Expt. 1, strain AF_2 produced a somewhat lower mortality 21 days after inoculation than did strains AF_1 and AF_3. Thus, while $9{\cdot}2 \times 10^6$ AF_2 spores proved fatal to only five of a group of 12 mice, $5{\cdot}8 \times 10^6$ AF_1 spores and $8{\cdot}4 \times 10^6$ AF_3 spores produced, respectively, 11 deaths ($P = 0{\cdot}01$) and 12 deaths ($P < 0{\cdot}0025$). It should be noted that strain AF_2 differed in growth characteristics from the other strains used: colonies which grew on Sabouraud's dextrose agar from well-separated spores did not exceed 1 mm. in diameter after 21 hr. incubation, whereas most colonies of the other four strains were at least twice as large; furthermore, after 2 and 3 days incubation the spores of strain AF_2 produced a greenish blue colour which was much lighter in shade than that produced by the other strains.

Thus it seemed unlikely that strain AF_1 had lost virulence during storage, and further information on this point was supplied by the next experiment.

Virulence of strain AF_1 after isolation from mice

Although the dose response after the autumn of 1970 was greatly reduced, small numbers of sporadic deaths occurred, sometimes surprisingly soon after inoculation, in mice treated with low doses. To exclude the possibility that these deaths might have been due to a small proportion of virulent spores in a conceivably heterogeneous inoculum, AF_1 isolates from six such mice were examined. The animals chosen consisted of four mice which died after doses ranging from $0{\cdot}7 \times 10^6$ to $0{\cdot}1 \times 10^6$ spores, and two of five mice which died from a group of 50 mice inoculated with $1{\cdot}3 \times 10^6$ spores.

Each isolate was purified and, 10 days after isolation, spore suspensions prepared in the usual way were inoculated intravenously into groups of eight SA mice. A further group of 12 mice received spores grown from the original, deep-frozen AF_1 cultures. The doses ranged from $0{\cdot}9 \times 10^6$ to $1{\cdot}7 \times 10^6$, and reference to Table 2 and the related text shows that, before the end of August 1970, such doses would have killed almost all mice within 8 days.

The results given in Table 4 show that after a single passage through mice strain AF_1 did not differ significantly in virulence from the original strain. Since growth without multiplication of the organism occurs in the depth of tissues, no attempt was made to raise virulence by repeated passage.

Table 5. *Effect of environment and age on resistance of mice to* Aspergillus fumigatus

Expt.	Days after intravenous inoculation	Dose of spores ($\times 10^6$)	Deaths of SA mice in four separate experiments: Mice bred by supplier A	Mice bred at Nuffield Institute	Significance (P) of difference in mortality between mice bred on different premises
			♂ mice weighing 20 g.	♂ mice weighing 10 g. aged 3 weeks	
A	6	5·6	2/15	12/12	< 0·00005
		1·4	0/15	8/12	< 0·0005
	28	5·6	12/15 (3)	12/12	NS
		1·4	2/15 (7)	*11/12 (1)	< 0·0001
			♀ mice weighing 22 g.	♀ mice weighing 26 g. aged 6 weeks	
B	4	5·3	0/15	4/12	< 0·017
		1·3	0/15	0/12	NS
	6	5·3	6/15	6/12	NS
		1·3	2/15	1/12	NS
	28	5·3	15/15	12/12	NS
		1·3	8/15 (3)	9/12 (3)	NS
			♀ mice weighing 21 g.	♀ mice weighing 30 g. aged 9 weeks	
C	6	5·3	5/15	11/12	< 0·003
		1·3	1/15	4/12	NS
	28	5·3	11/15 (3)	12/12	NS
		1·3	3/15 (9)	11/12 (1)	< 0·0005
			♂ mice weighing 20 g.	♂ mice weighing 37 g. aged 13 weeks	
D	6	1·6	1/15	6/22	NS
	10	1·6	2/15	12/22	< 0·0125
	28	1·6	3/15 (8)	15/22 (7)	< 0·005

NS = not significant.

Figures in parentheses indicate survivors showing active *A. fumigatus* lesions at slaughter, 28 days after infection.

* Result complete by 10th day.

Effect of environment and age on resistance

In view of the evidence that AF_1 had not lost virulence, it seemed certain that the resistance of the SA mice must have increased. As already explained, the circumstances indicated that increased resistance could not have resulted from genetical factors. The importance of environment is apparent from the next experiment.

Adult SA breeding mice were purchased from supplier A. Their offspring, bred at the Institute, were challenged with strain AF_1 immediately after weaning at the age of 3 weeks (mean weight 10 g.) and also at the ages of 6 weeks (weight 26 g.), 9 weeks (weight 30 g.) and 13 weeks (weight 37 g.). On each of these four occasions, mice weighing 20–22 g. were obtained from supplier A 24 hr. earlier and challenged simultaneously (Table 5).

As judged by mortality, mice bred at the Institute were more susceptible than

20–22 g. mice obtained from supplier A; this was so not only when they were smaller and younger than the purchased mice, but also when they were larger and older. The difference was very clear in each test except the second (Expt. B, Table 5), in which there was merely a slight difference in survival time; in this instance, the mortality of the purchased mice may have indicated a temporary upward fluctuation in the normally low susceptibility of the 20 g. SA mice, to a degree which was unique in the 15 experiments carried out after the autumn of 1970. It is clear from the cultures made on slaughtered survivors that Institute-bred mice, unlike those bred by supplier A, invariably became infected after inoculation of 10^6 to 2×10^6 spores.

The early onset of symptoms and the mortality pattern of Institute-bred mice aged 3 weeks (Expt. A, Table 5) indicated high susceptibility similar to that consistently found in adult SA mice before the end of August 1970 (see Table 2 and related text). Institute-bred mice of older age groups were probably less susceptible, and a further experiment on age and resistance was carried out using mice bred by supplier A.

Fifteen SA males immediately after weaning at 3 weeks (mean weight 11·7 g.) and 15 older males (mean weight 20 g.) were inoculated with $1{\cdot}6 \times 10^6$ spores. Total deaths in the 11·7 g. mice 6, 10 and 28 days later were 2, 3 and 12 respectively; the corresponding figures for deaths of 20 g. mice were 1, 2 and 3. Thus, the newly weaned mice were clearly more susceptible than the older animals ($P < 0{\cdot}002$ on day 28), but apparently less susceptible than the newly weaned mice bred earlier at the Nuffield Institute (Expt. A, Table 5).

Comparison of the susceptibility of mice from seven different sources

Finally, it seemed of interest to investigate the range of susceptibility to *A. fumigatus*, strain AF_1, shown by mice from a number of sources in the south-east of England. In one experiment, SA mice were compared with SB, SC, SD and SE_1 mice by inoculating various dilutions of spore suspension prepared from *A. fumigatus* strain AF_1. In a second experiment, carried out 14 weeks later, SA mice were compared with the specific pathogen free SE_2 and SF mice. The details of experimental design and the results are given in Table 6.

Expt. 1 revealed no clear differences between mice from five different sources in terms either of mortality 3 weeks after infection, or of active lesions in survivors killed at 3 weeks. Slight differences nevertheless occurred: for example, on the fourth day after inoculation, deaths produced in groups of eight animals by the 1/5, 1/10 and 1/40 inocula were respectively, 6, 4 and 1 in SD mice, but only 1, 0 and 0 in SE_1 mice and 2, 0 and 0 in SA mice. Expt. 2 revealed no differences in the susceptibility of SA and SE_2 mice, but the SF animals were clearly less resistant as judged by mortality 33 days after infection ($P < 0{\cdot}005$ at the 1/10 dose level). This difference in susceptibility was much less dramatic than that resulting from the natural increase in resistance of the SA mice (see Table 2). The greater susceptibility of SF mice is unlikely to have been related to their specific pathogen free status, since the more resistant SE_2 mice were also specific pathogen free. In general, $1{\cdot}4 \times 10^6$ spores produced low mortality in the experiments.

Table 6. *Comparison of the susceptibility of mice from seven different sources to* Aspergillus fumigatus, *strain* AF_1

Dilutions of Brown's no. 19 spore suspension (strain AF_1)	Deaths in mice from seven different sources							
	Expt. 1: results recorded 21 days after inoculation					Expt. 2: results recorded 33 days after inoculation		
	SA	SB	SC	SD	SE_1	SA	SE_2	SF
1/5	6/8 (2)	7/8 (1)	8/8	8/8	5/8 (3)	11/12 (1)	10/12 (2)	6/6
1/10	5/8 (3)	5/8 (3)	8/8	5/8 (3)	5/8 (3)	4/12 (5)	6/12 (6)	12/12
1/20	2/8 (2)	N	N	N	N	3/12 (4)	1/12 (9)	5/12 (6)
1/40	0/8 (5)	1/8 (6)	3/8 (4)	3/8 (5)	2/8 (4)	1/12 (8)	2/12 (9)	6/12 (5)
1/80	1/8 (4)	1/8 (4)	1/8 (6)	5/8 (2)	1/8 (4)	3/12 (3)	3/12 (4)	1/12 (8)
1/160	N	N	N	N	N	0/12 (3)	0/12 (8)	3/12 (5)
1/640	1/10 (4)	1/10 (1)	0/10 (0)	0/10 (3)	0/10 (1)	N	N	N

Figures in parentheses indicate number of survivors showing active *A. fumigatus* lesions on slaughter.

In both Expts 1 and 2, the 1/40 doses contained $1{\cdot}4 \times 10^6$ spores.

N = not done.

DISCUSSION

The use of mice provided a sensitive system for studying both experimental and natural resistance. Slight differences in susceptibility were revealed only by variations in survival time, but greater differences gave rise in addition to differences in infection rate. Challenge doses within an optimum range showed that sub-lethal doses of spores given intravenously could produce resistance of mice to *A. fumigatus*. Although this resistance was stronger when the initial dose was large enough to produce pathogenic effects in a proportion of animals, it also occurred after a dose too small to result in established infections.

Repeated observations over a period of 34 months revealed a single, very striking decrease in dose–response of the SA mice to *A. fumigatus*, strain AF_1. This was shown by evidence of a necessarily indirect or circumstantial nature to be due not to any decrease in virulence of strain AF_1, but to an increase in resistance of the mice, resulting from environmental and not genetical factors. Although the experiments of Scholer (1959) were carried out under slightly different conditions, it is interesting that the dose response which he observed resembled that initially shown by the SA mice. It is strange that, after the resistance of the SA mice suddenly increased, a search for highly susceptible mice from a considerable number of other sources in the south-east of England was unsuccessful, although minor differences in susceptibility between mice of different origin were observed. It is also interesting that, before the autumn of 1970, mice bred by supplier A were highly susceptible even after being kept for as long as 7 weeks at the Nuffield Institute between purchase and inoculation; yet subsequently, SA mice bred at the Institute appeared to possess susceptibility of a degree which was intermediate, though clearly greater than that of mice of the same stock bred by

supplier A. These circumstances indicate that, after the autumn of 1970, environmental resistance-producing factors may have been operating simultaneously, though to varying degrees, on a number of premises housing laboratory mice in south-east England. Unfortunately, mice from breeders other than supplier A were not examined before the autumn of 1970. In view of Scholer's (1959) findings concerning dose-response, it seems justified to suggest that the environmental factors which brought about the increase in resistance of SA mice to strain AF_1 also produced resistance to at least some, if not all, of the 4 additional strains used.

There is no proof as to which of the many possible environmental factors was responsible for the increase in resistance of the SA mice, but the following points may be pertinent. *A. fumigatus* is a ubiquitous organism. The concentration of fungal spores in the air is known to vary greatly both from place to place and from time to time (Hyde, Richards & Williams, 1956; Baruah, 1961; Noble & Clayton, 1963) and certain materials such as hay, particularly if mouldy, can provide abundant sources of fungal spores, many of which are of the genus *Aspergillus* (Gregory & Lacey, 1963*a*, *b*; Lacey & Lacey, 1964). Using an experimental system which was admittedly highly artificial, the present study showed that previous experience of the organism could result in a degree of resistance. Though not proved, it seems probable that the sudden increase in the resistance of the SA mice was due to a change in the degree of environmental contact with *A. fumigatus* or related fungi, living or dead, or to their metabolites. Such contact might have occurred either by inhalation or by ingestion in foodstuffs, and further research along these lines is indicated. The difference in susceptibility of mice of the same stock bred on different premises may have had a similar basis. Mice immediately after weaning at the age of 3 weeks were more susceptible than young adults of the same stock bred on the same premises, but the susceptibility of newly weaned mice appeared to be by no means uniform under all conditions. The lower resistance of 3-week-old mice may have been due to lack of specific immunity or to physiological factors.

It seems likely that environmental factors such as those which influenced the susceptibility of mice to artificial infection also operate in relation to other mammals and birds. This might have an important bearing on the occurrence and pathogenesis of the natural diseases produced by *A. fumigatus*.

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