INFECTION AND IMMUNITY, Nov. 1975, p. 987-992

Vol. 12, No. 5
Printed in U.S.A.

Chitin Assay Used to Demonstrate Renal Localization and Cortisone-Enhanced Growth of *Aspergillus fumigatus* Mycelium in Mice

PAUL F. LEHMANN* AND LES O. WHITE

Department of Microbiology, University of Birmingham, Birmingham, B15 2TT, England

Received for publication 29 April 1975

Aspergillus fumigatus mycelium in untreated mice (N-mice) and cortisone acetate-treated mice (C-mice) has been quantified by chemical assay of fungal chitin. Cortisone pretreatment rendered mice more susceptible to infection by *A. fumigatus* (mean lethal dose at 20 days, $\simeq 10^6$ for N-mice; $<10^4$ for C-mice). In both N- and C-mice there was renal localization of mycelial infection at conidial doses less than the mean lethal dose. At a conidial dose greater than the mean lethal dose, mycelial infection was found in the kidneys and brain of N-mice and in the kidneys, liver, and heart of C-mice. Chitin assay results showed that *A. fumigatus* mycelium grew more rapidly in C-mice. It is suggested that the resistance of N-mice to mycelial development may be an important mechanism whereby natural resistance to *A. fumigatus* is conferred.

Corticosteroid therapy may render man susceptible to systemic infections from opportunistic fungi such as *Candida albicans* and *Aspergillus fumigatus* (7, 14). The incidence of these infections is increasing; indeed recently 8 of 51 renal transplant recipients developed fatal aspergillosis (2).

Mice treated with cortisone have decreased resistance to fungal infection (11, 16); however, there have been few attempts to quantify the growth rate of the infecting fungi in vivo. Because of its filamentous nature, *A. fumigatus* has no reproducible infective unit, and this prevents easy interpretation of viable counts taken from homogenates of infected organs where spores, small pieces of hypha, and mycelial clumps will have equal value. In addition, because of its three-dimensional nature, mycelium in infected organs cannot easily be quantified by histological methods.

This paper describes the fate of *A. fumigatus* conidia in untreated mice (N-mice) and cortisone acetate-treated mice (C-mice). A chemical assay has been used to quantify the total mycelium that develops in susceptible organs. The assay (Fig. 1) was developed for problems in plant pathology (13) and estimates chitin, a material occurring in fungal cell walls but not in vertebrates. Chitin is converted to chitosan by the action of hot concentrated alkali. An aldehyde derivative of chitosan is made on reaction with HNO_2 and the aldehyde groups are assayed colorimetrically. A preliminary report of this work has been published (10).

MATERIALS AND METHODS

Inocula. *A. fumigatus* strain 2085, obtained from the Mycological Reference Laboratory, London School of Hygiene and Tropical Medicine, was grown on 2% malt extract agar for 48 h at 37 C. Conidia were suspended in Tween 80 (0.05% vol/vol) and the suspensions were shaken vigorously to collect clumps of conidia in the froth. Froth was discarded, and the unclumped conidia were counted and adjusted to the desired concentration. The viability of the conidia was >95%.

Cortisone treatment. Mice were given 5 mg of cortisone acetate (Cortisab, Boots Co., Nottingham, U.K.) subcutaneously, administered 48 h before injection of conidia unless otherwise stated.

Animal infection. Male, 5-week old LACA mice (SACI, Norwich, U.K.) were inoculated in the lateral tail vein with 0.1 ml of conidial suspension. At intervals after infection, mice were killed by cervical dislocation. Organs were removed aseptically.

Viable counts. Fresh whole organs were homogenized in 5 ml of distilled water. Aliquots (0.1 ml) of homogenate, or of serial dilutions, were plated onto 2% malt extract agar containing Crystamycin Glaxo (60 μg of benzylpenicillin and 100 μg of streptomycin/ml). Colonies were counted after 24 h of incubation at 37 C. The remaining homogenate was stored at −20 C for subsequent chitin assay.

Chitin assay. The chitin content of organ homogenates was assayed by a modification of a method described for the estimation of fungal mycelium in diseased tomatoes (13). The method, requiring the preparation and subsequent assay of chitosan, was as follows. Thawed homogenate was centrifuged (1,500 × *g*, 5 min, 20 C) in 15-ml graduated centrifuge tubes and the supernatant was discarded. The pellet was resuspended in 4 ml of sodium lauryl

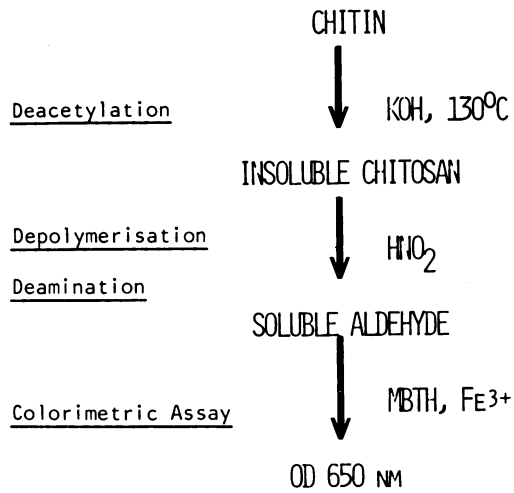

FIG. 1. *Assay of chitin in infected tissues (based on Ride and Drysdale [13]).*

sulfate (3% wt/vol) and heated (100 C, 15 min). After cooling, the tubes were recentrifuged and the supernatant was discarded. The pellet was washed once with distilled water, resuspended in 3 ml of KOH (120 g in 100 ml of water), and heated (130 C, 1 h). After cooling, 8 ml of ice-cold ethanol (75% vol/vol) was added and the tubes were shaken until the KOH and ethanol formed a single phase; if the latter failed to form unaided, a few drops of water were added. The tubes were kept in ice water for 15 min and 0.3 ml of Celite suspension (the supernatant left when 1 g of Celite 545 was mixed with 75% ethanol and left to stand for 2 min) was then added. The tubes were centrifuged (1,500 × *g*, 5 min, 2 C) and the pellet was washed once with ice-cold ethanol (40% vol/vol) and twice with ice-cold water. This pellet, containing insoluble chitosan, was stored at +4 C until a convenient time for subsequent assay.

Distilled water was added to the chitosan pellet to the 0.5-ml mark on the sample tubes. Since chitosan is assayed in relation to a known quantity of glucosamine, standards were set up containing 0.2 ml of water or 0.2 ml of glucosamine (10 μg/ml). To the samples, 0.5 ml of $NaNO_2$ (5% wt/vol) and 0.5 ml of $KHSO_4$ (5% wt/vol) were added; 0.2 ml of each solution was added to the standards. All tubes were gently mixed three times during 15 min and then centrifuged (1,500 × *g*, 2 min, 2 C). Two 0.6-ml volumes of supernatant from the sample tubes were taken and these, together with the standards, were brought to room temperature. To all tubes 0.2 ml of ammonium sulfamate (12.5% wt/vol) was added, and the tubes were mixed vigorously each minute for 5 min. Freshly made MBTH (0.2 ml, 50 mg of 3-methylbenzo-2-thiazolone hydrazone HCl monohydrate, obtained from Koch Light Labs, Coinbrook, Bucks., U.K., in 10 ml of water) was added and the tubes were heated (100 C, 3 min). After cooling, 0.2 ml of $FeCl_3 \cdot 6H_2O$ (0.83% wt/vol) was added and the optical density (650 nm) was measured 25 min later in a Unicam SP1800 spectrophotometer with microcells (2-cm light path). When necessary the colored solution was diluted with water to bring the optical density within the instrument's range.

Throughout this paper the chitin content of organs has been expressed as a glucosamine equivalent. In all assays uninfected organs which gave a slight color reaction were used as blanks. The chitin content of a test organ (X) is given by the equation $X = 5(A - B) \div (G - W)$ μg of glucosamine, where the optical density (650 nm) for the test organ is A, the uninfected control is B, the glucosamine standard is G, and the water standard is W.

Histology. Organs were fixed in 10% formal-saline and wax embedded. Sections were stained by the method of Gomori-Grocott (1).

Mycelial homogenate. Mycelium, from 3-day cultures grown in Czapek-Dox broth (37 C, orbital shaking at 150 rpm), was washed in distilled water and homogenized in a Griffith tube. The homogenate was stored at −20 C.

RESULTS

Reliability of the assay. Assay of various volumes of a homogenized suspension of mycelium grown in vitro showed that a linear relationship between the volume of mycelial homogenate and the chitin content existed over a wide range (Fig. 2). Variability between replicates was approximately 1 μg of glucosamine at all levels of chitin.

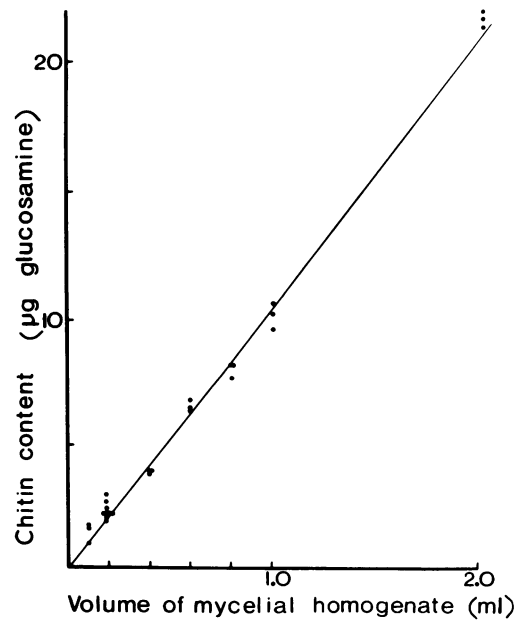

FIG. 2. *Relationship between observed chitin content and amount of fungal mycelium assayed. Points represent individual determinations which have been clumped horizontally when identical.*

Table 1 demonstrates that interfering substances in the organ homogenates were not found. There was no difference in the chitin content of aliquots of mycelial suspension when assayed in the presence or absence of organ homogenate (provided that adjustments were made for organ controls).

Fate of C- and N-mice injected with different doses of conidia. Groups of mice were injected with conidia, and at death or 20 days postinjection a viable count of fungus in their organs was made. The results (Table 2) showed a mean lethal dose (LD_{50}) for C-mice $<10^4$ and for N-mice $>10^6$ conidia. At low doses there was pronounced renal localization of viable fungus, though the animals did not necessarily die. Histology confirmed that mycelium developed in the kidneys.

Fate of conidia in C- and N-mice. When conidia were given at levels $<LD_{50}$ (N-mice, 10^5; C-mice, 3×10^3), viable fungus was cleared from all organs with only the kidneys remaining infected. Here, beyond day 2 the viable count subsequently rose and was associated with a positive chitin content (Table 3). Other organs showed no significant chitin. Histology showed mycelium in the kidneys 3 days postinfection.

When doses of conidia $>LD_{50}$ (N-mice, 5×10^6; C-mice, 10^5) were given, infection was not confined to the kidneys. In N-mice brain infection was found and in C-mice the liver and heart became infected, as shown by the development of a positive chitin content (Table 4) and the presence of mycelium (histology).

Figure 3 shows how viable counts and chitin content of livers were related in these C- and N-mice. In the livers of N-mice, no chitin was detectable at any time, even though 2 h postinjection the viable count was $>10^6$. In C-mice, however, a rise in viable count occurred after 2 days and was associated with development of chitin.

Effect of cortisone on established mycelium. The action of cortisone on the growth of mycelium, previously established in the mouse, was investigated. N-mice were injected with 5×10^5 conidia and left for 4 days. This allowed the establishment of mycelium in the kidneys and the clearance of ungerminated conidia. At 4 days mice were randomly placed into three groups. One group (group 1) was killed immediately, one group (group 2) was left untreated and killed 3 days later, and the last group (group 3) was given 5 mg of cortisone and killed 3 days later. The kidneys were individually assayed for chitin and viable fungus.

Mice that had been killed 7 days after infection (Fig. 4, groups 2 and 3) had more mycelium than the mice killed at 4 days (group 1); however, C-mice (group 3) developed more mycelium in the 3 days subsequent to the cortisone treatment that the N-mice (group 2) as measured by both viable count and chitin assay (Fig.

TABLE 1. *Chitin content of mycelial suspension assayed in the presence or absence of an organ homogenate*

Organ	Chitin content[a] (μg of glucosamine)	
	Mycelium alone	Mycelium plus organ homogenate[b]
Kidney	5.3 ± 0.3	5.0 ± 0.2
Brain	5.3 ± 0.3	5.2 ± 0.4
Spleen	4.9 ± 0.8	5.7 ± 0.5
Lung	5.6 ± 0.4	5.4 ± 0.1
Heart	5.4 ± 0.4	4.9 ± 0.4
Liver	5.5 ± 0.5	5.0 ± 1.0

[a] Mean and standard error of six replicates.
[b] Organ homogenate blanks have been subtracted.

TABLE 2. *Mortality at 20 days and organ infection of dead mice or mice surviving to 20 days after intravenous challenge with various doses of Aspergillus fumigatus conidia*

Mice	Conidial dose	Mortality[a] (20 days)	No. of mice with viable fungus in:					
			Kidney	Liver	Heart	Spleen	Lung	Brain
N	10^7	4/4	4	3	2	2	2	2
	10^6	1/5	5	0	0	0	0	0
	10^5	0/6	4	0	0	0	0	0
C	10^5	6/6	6	6	6	1	1	0
	10^4	6/6	6	6	2	0	0	0
	10^3	1/6	3	1	1	0	0	0
	10^2	1/6	3	0	0	0	0	0

[a] Mortality given as number of dead/number injected.

TABLE 3. *Viable count and chitin content (as micrograms of glucosamine) of whole organs from mice killed at intervals after intravenous inoculation of a low dose of A. fumigatus conidia*[a]

Organ	Mouse no.	Viable counts (days postinfection)					
		0.1	1	2	3	7	14 or 10[b]
N-mice							
Brain	1	100 (—)[c]	0	0	0	0 (—)	0
	2	0 (—)	0	0	100	0 (—)	0
	3	0 (—)	0	200	0	200 (—)	0
Heart	1	900 (—)	200	0	0	0 (—)	0
	2	100 (—)	200	0	0	0 (—)	0
	3	700 (—)	200	0	100	0 (—)	0
Kidneys	1	19,000 (—)	1,300 (—)	100 (—)	400 (—)	2,300 (6.5)	700 (0.8)
	2	9,500 (—)	800 (—)	100 (—)	1,800 (0.6)	2,800 (4.8)	2,900 (9.3)
	3	12,000 (—)	1,700 (—)	700 (—)	100 (—)	700 (1.7)	2,800 (NT)[d]
Liver	1	400,000 (—)	1,300	100	0	0 (—)	0
	2	200,000 (—)	1,500	100	200	0 (—)	0
	3	460,000 (—)	2,900	400	100	0 (—)	0
Lungs	1	1,500 (—)	100	0	0	0 (—)	0
	2	2,700 (—)	0	0	0	0 (—)	0
	3	3,500 (—)	100	0	0	0 (—)	0
Spleen	1	22,000 (—)	4,300	200	100	0 (—)	0
	2	12,000 (—)	4,500	900	100	0 (—)	0
	3	40,000 (—)	5,600	1,900	100	0 (—)	0
C-mice							
Brain	1, 2, 3	0[e]	0[e]	0[e]	0[e]	0[e]	0[e]
Heart	1, 2, 3	0[e]	0[e]	0[e]	0[e]	0[e]	0[e]
Kidneys	1	90 (—)	40 (—)	70 (—)	2,000 (3.7)	20,200 (10.0)	2,400 (4.0)
	2	140 (—)	60 (—)	70 (—)	730 (1.1)	9,500 (9.6)	7,300 (22.2)
	3	70 (—)	10 (—)	300 (—)	800 (2.3)	18,900 (20.5)	2,400 (4.3)
Liver	1	1,200 (—)	10	25	100	0 (—)	400 (—)
	2	1,000 (—)	0	50	25	0 (—)	0 (—)
	3	1,600 (—)	75	50	0	0 (—)	0 (—)
Lungs	1	120	0	0	0	0	0
	2	170	0	0	0	0	0
	3	120	0	0	0	0	0
Spleen	1	50	0	0	0	0	0
	2	40	0	0	0	0	0
	3	50	0	0	0	0	0

[a] Doses: N-mice, 5×10^5; C-mice, 3×10^3.
[b] N-mice 14 days, C-mice 10 days.
[c] —, Insignificant chitin.
[d] NT, Sample lost.
[e] All replicates zero.

4). The differences were statistically significant (99%).

DISCUSSION

The chitin assay quantifies fungal cell wall material present in infected organs and thus may estimate total fungal mass. The assay will reliably detect an amount of mycelium having a chitin content equivalent to 1 μg of glucosamine or greater (Fig. 2). Unlike a viable count, the assay is insensitive to large numbers of conidia, for even 2.4×10^6 conidia do not contain a measurable level of chitin (Fig. 3).

Estimation of fungal material in vivo is probably the best measure of infection in the case of a mycelial fungus which has no standard infective unit. The chitin content of the mycelium per gram (dry weight) of *A. fumigatus* and *Fusarium oxysporum* grown in vitro remained fairly constant until autolysis began (unpublished data; 13). However, even if the chitin content of the mycelium did not remain constant in vivo, or differed in N- and C-mice, an increase in the chitin content of an infected organ would indicate an increase in fungal biosynthetic metabolism. This assay should be suitable for other mycelial animal pathogens, since they belong to taxonomic groups which have cell wall chitin (3).

TABLE. 4. *Chitin content of organs 0.1 and 4 days postinfection with a high dose of A. fumigatus conidia*[a]

Organ	Chitin content (μg of glucosamine)		
	N- and C-mice[b] (0.1 days)	N-mice[b] (4 days)	C-mice[b] (4 days)
Kidneys	(—)[c]	6.8, 64.1, 2.0	21.8, 67.4, 67.1
Brain	(—)	1.0, 1.9, 0.5	(—)
Spleen	(—)	(—)	(—)
Lungs	(—)	(—)	(—)
Heart	(—)	(—)	2.7, 2.1, 3.7
Liver	(—)	(—)	1.6, 1.0, 8.7

[a] Doses: N-mice, 5×10^6; C-mice, 10^5.
[b] Results for three mice in each group.
[c] —, Insignificant chitin.

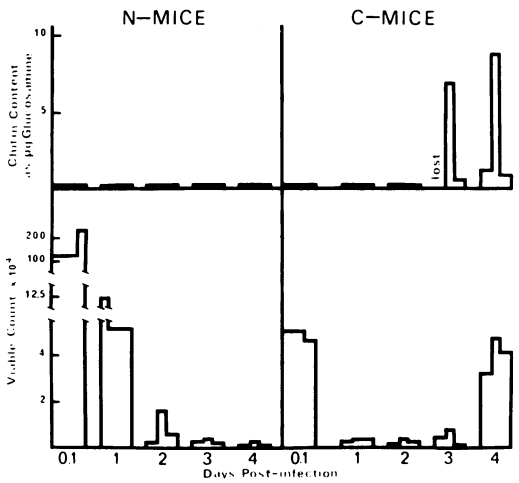

FIG. 3. *Viable count and chitin content of livers in N- and C-mice receiving a high dose of conidia (doses: N-mice, 5×10^6; C-mice, 10^5). Results for three mice killed at each time point.*

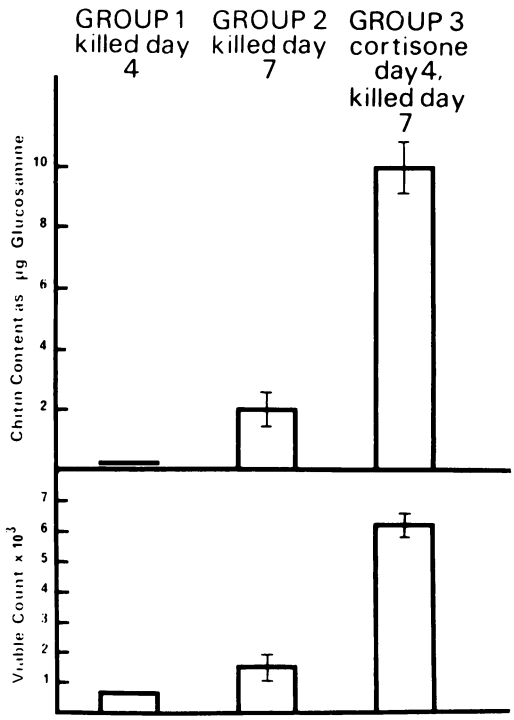

FIG. 4. *Effect of cortisone, given 4 days postinfection with 5×10^5 conidia, on the chitin content and viable count from mouse kidneys. (Each column represents the mean of 12 kidneys with standard error bars shown.)*

In mice there is a rapid clearance of fungus from organs immediately after injection of conidia and, at low conidial doses, viable fungus is only retained in the kidneys. In this respect *A. fumigatus* differs from *A. clavatus* (6), *A. bisporus* (9), and *Absidia ramosa* (18), whose viable conidia or resting spores remained ungerminated in various organs for extended periods and failed to cause invasive infection.

Renal localization of infection in untreated mice, after conidia have been introduced intravenously, has been reported by other (15, 17) but not as we have shown here in cortisone-treated mice. In man, *Aspergillus* spp. (including *A. fumigatus*) usually cause pulmonary disease, though in certain situations they infect the kidneys. Thus *A. fumigatus* infection localized in the kidney of a diabetic heroin addict (4) and 55% of the patients who developed *Aspergillus* spp. endocarditis after open heart surgery also had kidney involvement (8). In both types of patient the fungus might be expected to enter the bloodstream directly and to bypass the lungs.

The factors that determine the susceptibility of the mouse kidneys after intravenous administration of conidia are not clear. Susceptibility to infection may be linked to rapid germination of conidia with subsequent mycelial growth overcoming the local host defenses (5, 16, 19). In-

deed, spores of *A. flavus* and *Absidia ramosa*, pregerminated in vitro, are more pathogenic than dormant spores (16, 18). We have shown (manuscript in preparation) that the majority of *A. fumigatus* conidia germinate in the kidneys of both untreated and cortisone-treated mice by 24 h postinjection. Therefore, any resistance occurring after this time must be directed against developing mycelium if it is to be at all effective. Our results have demonstrated that mycelium grows more rapidly in cortisone-treated mice (receiving cortisone 4 days postinfection) than in untreated mice. It therefore appears that certain host defenses to mycelial development exist in the kidney and are inhibited by the cortisone treatment. The nature of these host defenses is not clear. Injected cortisone has been reported (12) to inhibit the mechanisms whereby phagocytosed *A. flavus* conidia are prevented from germinating in mouse alveolar macrophages; however, there have been no reports of macrophages inhibiting the growth of mycelium, which will almost certainly fail to be phagocytosed on account of its size. An extracellular mode of action would be expected for defenses against developing fungal mycelium.

Thus, cortisone-sensitive resistance to mycelial growth is naturally present in the mouse kidney and may be an important aspect of the resistance of mice to fungal infection. It may be that the insusceptibility of other organs (e.g., the liver and heart) is similarly due to a defense against hyphal growth as well as any possible inhibition of spore germination.

ACKNOWLEDGMENTS

This work was supported by a grant from the Medical Research Council. We would like to thank J. P. Ride for discussions concerning the chitin assay.

LITERATURE CITED

1. Austwick, P. K. C. 1965. Pathogenicity, p. 82–126. *In* K. A. Raper and D. I. Fennell (ed.). The genus *Aspergillus*. The Williams and Wilkins Co., Baltimore.
2. Bach, M. C., A. Sahyoun, J. L. Adler, R. M. Schlesinger, J. Breman, P. Madras, F. P'eng, and A. P. Monaco. 1973. High incidence of fungus infections in renal transplantation patients treated with antilymphocyte and conventional immunosuppression. Transplant. Proc. 5:549–553.
3. Bartnicki-Garcia, S. 1968. Cell wall chemistry, morphogenesis, and taxonomy of fungi. Annu. Rev. Microbiol. 22:87–108.
4. Chmel, H., and M. H. Grieco. 1973. Cerebral mucormycosis and renal aspergillosis in heroin addicts without endocarditis. Am. J. Med. Sci. 226:225–231.
5. Eades, S. M., and M. J. Corbel. 1973. The effect of soluble bovine placental extracts on the growth of fungi implicated in bovine mycotic abortion. Proc. Soc. Gen. Microbiol. 1:27.
6. Ford, S., and L. Friedman. 1967. Experimental study of the pathogenicity of aspergilli for mice. J. Bacteriol. 94:928–933.
7. Frenkel, J. K. 1962. Role of corticosteroids as predisposing factors in fungal diseases. Lab. Invest. 11:1192–1208.
8. Kammer, R. B., and J. P. Utz. 1974. *Aspergillus* species endocarditis. Am. J. Med. 56:506–521.
9. Kwon-Chung, K. J. 1971. A new pathogenic species of *Aspergillus*. Mycologia 63:478–489.
10. Lehmann, P. F., L. O. White, and J. P. Ride. 1974. Aspects of systemic *Aspergillus fumigatus* infection in untreated and immunosuppressed mice: use of a chitin assay to measure fungal growth. Proc. Soc. Gen. Microbiol. 2:30.
11. Louria, D. B., and H. G. Browne. 1960. The effect of cortisone on experimental fungal infections. Ann. N. Y. Acad. Sci. 89:39–46.
12. Merkow, L. P., S. M. Epstein, H. Sidransky, E. Verney, and M. Pardo. 1971. The pathogenesis of experimental pulmonary aspergillosis. Am. J. Pathol. 62:57–68.
13. Ride, J. P., and R. B. Drysdale. 1972. A rapid method for the chemical estimation of filamentous fungi in plant tissue. Physiol. Plant Pathol. 2:7–15.
14. Rifkind, D., T. L. Marchioro, S. A. Schneck, and R. B. Hill. 1967. Systemic fungal infections complicating renal transplantation and immunosuppressive therapy. Am. J. Med. 43:28–38.
15. Scholer, H. J. 1959. Experimentelle Aspergillose der Maus (*Aspergillus fumigatus*) und ihre chemotherapeutische Beeinflussung. Schweiz. Z. Pathol. Bakteriol. 22:564–576.
16. Sidransky, H., S. M. Epstein, E. Verney, and C. Horowitz. 1972. Experimental visceral aspergillosis. Am. J. Pathol. 69:55–70.
17. Smith, G. R. 1972. Experimental aspergillosis in mice: aspects of resistance. J. Hyg. (London). 70:741–754.
18. Smith, J. M. B., and R. H. Jones. 1973. Localisation and fate of *Absidia ramosa* spores after intravenous inoculation of mice. J. Comp. Pathol. 83:49–55.
19. White, L. O., and H. Smith. 1974. Placental localisation of *Aspergillus fumigatus* in bovine mycotic abortion: enhancement of spore germination *in vitro* by foetal tissue extracts. J. Med. Microbiol. 7:27–34.