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Experimental phycomycosis in mice; examination of the role of acquired immunity in resistance to *Absidia ramosa*

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SUMMARY

Attempts were made to stimulate acquired immunity to experimental *Absidia ramosa* infection in mice. Unprotected animals inoculated with large doses of *A. ramosa* spores frequently developed acute phycomycosis of the central nervous system. Mice previously exposed to sub-lethal doses of spores showed a high resistance to subsequent challenge with *A. ramosa*. No consistent increase in resistance was observed in mice vaccinated with killed *A. ramosa* spores, hyphal walls, intracellular mycelial antigens or various combinations of these, with Freund's incomplete adjuvant.

Antibodies to soluble mycelial antigens were inconsistently present in the sera of mice vaccinated with sub-lethal doses of viable spores. They were generally present in the sera of animals vaccinated with mycelial extracts or hyphal walls but not killed spores.

Delayed hypersensitivity reactions to *A. ramosa* mycelial antigens could usually be elicited by intradermal tests in mice exposed to viable spores but irregularly in those vaccinated with non-viable preparations. Positive reactions were also frequently given by older mice not deliberately exposed to *A. ramosa.*

Although mice previously exposed to viable *A. ramosa* spores were highly resistant to intravenous or intracerebral challenge with this fungus, they were more likely to develop persistent local granulomata on subcutaneous injection of spores than were unvaccinated animals.

INTRODUCTION

Sporadic infections by normally saprophytic fungi are widely recognized as causes of disease in both man and animals (Ainsworth & Austwick, 1959; Emmons, Binford & Utz, 1970; Baker, 1971). In many instances the fungi implicated are common environmental organisms to which the majority of animals exposed show a high degree of natural resistance. Although some information is available on the mechanisms of immunity to the more virulent primary fungal pathogens such as *Histoplasma capsulatum, Coccidioides immitis* and *Blastomyces dermatitidis* (Kong & Levine, 1967) little is known of the basis of resistance to opportunistic fungal pathogens.

Recently, using infection with the phycomycete *Absidia ramosa* (*Absidia corymbifera*) as an experimental model for the study of immunity to opportunistic

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fungal pathogens, Corbel & Eades (1975) concluded that the natural resistance of mice to this fungus was largely dependent upon non-specific processes. Thus their observations on the effects of treatment with a variety of immunosuppressive agents indicated that phagocytic cells and possibly non-specific serum factors were largely instrumental in suppressing the germination and subsequent development of *A. ramosa* spores *in vivo*.

During the course of these studies it was observed that the proportion of mice resistant to challenge with standard doses of spores tended to increase with the age of the animals. This increased resistance was evidently not related solely to increased size or body weight as it also applied to inoculation via the intracerebral route. This suggested, *inter alia*, that the mice may have acquired immunity to *A. ramosa*, possibly as a result of environmental exposure to this or antigenically related organisms.

In the present study the influence of active immunization on susceptibility to *A. ramosa* infection has been examined in an attempt to assess the role of acquired immunity in resistance to phycomycosis.

MATERIALS AND METHODS

Mice

C3H mice between 19 and 21 days old at the beginning of the experiment were used. They were kept in groups of six animals to a cage and allowed free access to food and water. To minimize exposure to environmental fungi, food was restricted to a commercial sterilized mouse diet and the mice were bedded on sterilized wood shavings. During the course of the experiment the animals were excluded from contact with hay, straw or fresh vegetable matter.

Fungal strains

A. ramosa strain V.73/8 was used throughout. The origins of this strain have been described previously (Corbel & Eades, 1973). Spore suspensions were prepared and counted as described elsewhere (Corbel & Eades, 1975). *A. ramosa* mycelium was obtained by rapid growth of the fungus in stirred cultures of Czapek-Dox broth supplemented with 1%, w/v, yeast extract.

Fungal antigens

A. ramosa mycelial extract was produced by disrupting washed, freshly grown *A. ramosa* mycelium in an X-press (Biotec, Croydon) according to the procedures described by Corbel, Pepin & Millar (1973).

A. ramosa hyphal walls were prepared from the deposit obtained after centrifuging X-press disrupted mycelium at 10,000 ***g*** for 20 min. After decantation of the supernatant containing the soluble antigens, the deposit was washed by 6 cycles of centrifugation in phosphate buffered saline (PBS; 0·15 M-NaCl, 0·01 M phosphate buffer, pH 7·2), followed by 2 cycles of centrifugation in distilled water, all at 10,000 ***g*** for 20 min. The washed hyphal walls were finally suspended in PBS to a concentration of 10% wet weight per unit volume.

Fungal spores were obtained from cultures of *A. ramosa* grown on 2½%, w/v, malt extract agar slopes at 25° C. for 7 or 8 days. The spores were harvested in

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PBS by shaking with glass beads. Counts were made as described elsewhere (Corbel & Eades, 1975). Standard viable spore suspensions for inoculation were prepared by resuspension of the stock preparations to the required concentration in PBS. These were kept at 4° C. until required and used within 4 hr. of preparation.

Inactivated spores were produced from standard spore suspension by adding formalin to a final concentration of 0·5% formaldehyde and incubating at room temperature (17–19° C.) overnight.

For injection, inactivated spores and hyphal walls were suspended in PBS to a final concentration of 10%, w/v. The mycelial extract was used at a dry weight concentration of 50 mg. per ml. Mixtures of spores and hyphal walls or spores, hyphal walls and mycelial extract were made by mixing equal volumes of the standard preparation of each component. All of these preparations were emulsified with an equal volume of Freund's incomplete adjuvant.

Immunization procedures

Vaccination with non-viable fungal preparations. Groups of mice between 19 and 21 days old were given subcutaneous injections of 0·2 ml. volumes of inactivated spores, hyphal walls, mycelial extract or mixtures of spores and hyphal walls or spores, hyphal walls and mycelial extract, in Freund's incomplete adjuvant. A control group received similar injections of 0·2 ml. volumes of PBS emulsified in an equal volume of incomplete adjuvant.

Vaccination with viable spores. Groups of mice between 19 and 21 days old were given doses of 5×10^4 viable *A. ramosa* spores by the intravenous route. A proportion of the inoculated animals, usually *ca.* 10%, developed acute phycomycosis and died within 8 days. The survivors were retained and challenged with graded doses of *A. ramosa* spores 4 weeks after the initial inoculation. The animals in these groups were challenged with *A. ramosa* spores between 4 and 5 weeks after vaccination, *vide infra*.

Assessment of immune status

Vaccinated mice and groups of unvaccinated control animals of the same age kept under similar conditions were challenged by intravenous injection of 0·1 ml. volumes of *A. ramosa* spores suspensions containing 5×10^5, 5×10^6 or 5×10^7 viable units.

Alternatively, some animals were challenged by intracerebral injection of doses of 125 viable *A. ramosa* spores contained in 0·025 ml. volumes of PBS.

The challenged mice were observed over a period of 6 months. All animals dying were autopsied and tissues removed for mycological and histological examination. Samples of brain, heart, liver, lung, spleen and kidney tissue were cultured on $2\frac{1}{2}$% malt agar containing 20 units of penicillin G and 40 μg. of streptomycin per ml. Other samples of these tissues were examined by direct microscopy after digestion in 40%, w/v, KOH.

Portions of tissue fixed in 10%, v/v, buffered formalin were paraffin-embedded and sectioned. Sections were stained with haematoxylin and eosin or by the periodic acid–Schiff's–light green (PAS) procedure (Emmons *et al.* 1970).

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Serological tests

Immunodiffusion tests were done essentially according to the method of Ouchterlony (1953). The reagent wells were 3 mm. in diameter and cut in an isometric pattern with centres 5 mm. apart. The diffusion medium was that employed in a previous study (Corbel *et al.* 1973). *A. ramosa* mycelial extract was used as antigen at concentrations of 25 and 50 mg./ml.

Latex agglutination tests were performed essentially according to the procedure described by Amos (1970). The latex was sensitized with *A. ramosa* mycelial extract at an initial concentration of 50 mg./ml. Control tests were run in parallel using unsensitized latex suspensions.

Intradermal tests for delayed hypersensitivity

These were done by injecting volumes of *ca.* 0·02 ml. of various concentrations of mycelial extract, ranging from 500 μg./ml. to 50 mg./ml. into the hind foot pads of mice. Control tests were done by injecting 0·15 M-NaCl into the pads of the opposite foot. After 24 hr. the mice were killed and the feet excised and fixed in 10%, v/v, buffered formalin. Wax-embedded sections were cut and stained with haematoxylin and eosin and the hypersensitivity reactions assessed histologically.

Determination of the cellular response to fungal infection

This was done in a similar manner to the intradermal tests except that 0·1 ml. volumes of a suspension containing *ca.* 10^6 viable *A. ramosa* spores were injected into the subcutaneous tissue of the mice. Control tests were done with similar volumes of sterile PBS. At various intervals after injection selected animals were killed and the test sites excised and examined histologically as described for intradermal tests.

RESULTS

The response of unvaccinated mice to inoculation with viable A. ramosa *spores*

After intravenous injection of graded doses of *A. ramosa* spores a proportion of the animals in the control groups developed typical signs of phycomycosis of the central nervous system. Thus between the third and the eighth day after inoculation the affected animals became lethargic, refused food and water and then developed a continuous circling movement. This phase was followed by collapse or convulsions, rapidly succeeded by death. All affected animals died within 8 hr. of the onset of signs of the disease. The precise proportion of animals affected varied somewhat between individual experiments but was related to the spore dose. Thus between 70 and 90% of those receiving 5×10^7 spores were affected whereas the proportions were *ca.* 50 and 20–30% respectively for those receiving 5×10^6 and 5×10^5 spores.

At post-mortem examination gross lesions were not seen but microscopic lesions containing fungal mycelium were found in the brain and kidneys of the affected animals. Fungal spores were readily visible in PAS-stained sections of liver, lung and spleen (Pl. 1, fig. 1). They were rarely seen in sections of brain or kidney, even at the sites of active lesions. *A. ramosa* was recovered from cultures

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of the liver, lungs, spleen, kidneys and usually the brains of affected animals. Occasionally *A. ramosa* was not isolated on culturing the brain although fungal hyphae were detected on microscopical examination.

Histological examination of H & E and PAS stained sections of brain from affected mice revealed typical acute phycomycotic lesions. Within these, lengths of non-septate, relatively straight mucoraceous hyphae were visible. These were usually surrounded by infiltrations of predominantly mononuclear cells, although polymorphonuclear leucocytes were prominent in some lesions (Pl. 1, figs. 2, 3).

Examination of H & E stained sections of renal tissue revealed the presence of lesions in the medulla and pelvis of the kidney. These consisted of accumulations of polymorphonuclear and mononuclear cells surrounding damaged tubules Pl. 1, fig. 4). Fungal mycelium was not easily visible in these preparations but was readily apparent in PAS stained sections. The hyphae were of typical mucoraceous type.

Although fungal spores could be seen in PAS stained sections of other tissues, particularly spleen, liver and lungs, signs of active fungal growth or evidence of inflammatory cellular reaction were not apparent.

In those mice which did not develop acute phycomycosis but were killed 3 or 4 weeks after intravenous inoculation of *A. ramosa* spores, no lesions or fungal hyphae were detected in any organs other than the kidneys. In most of these animals the lesions were similar in histological appearance to those seen in acute infections although more circumscribed.

Precipitating antibodies to *A. ramosa* mycelial antigens were not detected in unvaccinated mice which had not been challenged with *A. ramosa* spores. Serum collected from these animals had reciprocal latex agglutination titres to *A. ramosa* antigens ranging from 1/2 to 1/16.

Delayed hypersensitivity reactions to mycelial antigens were also irregularly elicited in these animals. However, it was observed that the proportion of positive reactions increased with age. Thus over 90% of an uninoculated group of mice 6 months old produced intradermal reactions consistent with delayed hypersensitivity to *A. ramosa* antigens. These mice showed a high but not absolute resistance to *A. ramosa*. Thus intravenous doses of 5×10^7 spores produced acute lethal infection in about one third of those challenged.

Assessment of immunity to A. ramosa *in mice vaccinated with non-viable fungal preparations*

No conclusive evidence of any protective effect was apparent on challenging the groups of mice vaccinated with various *A. ramosa* preparations (Table 1). A similar proportion of animals developed lethal infection after intravenous injection of *A. ramosa* spores, whether vaccinated with diluent in adjuvant or with suspensions of inactivated spores, hyphal walls or mycelial extract, or mixtures of these in adjuvant. Similarly the mean times of death after challenge were similar for all of the groups. The gross and microscopic features of the acute infection were also similar for all animals irrespective of vaccination history.

The failure to confer protection against *A. ramosa* was not attributable to lack

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Table 1. *The effect of vaccination with various* A. ramosa *antigens on the susceptibility of mice to subsequent challenge with the fungus*

Group Number	Vaccine preparation	Challenge dose of *A. ramosa* spores	Number of deaths/number inoculated	Number of animals with fungal hyphae in the tissues of: Brain	Kidney	Other organs	Number of animals yielding cultures of *A. ramosa* from the tissues of: Brain	Kidney	Liver	Lung	Spleen	Bone marrow	Serological activity: Immuno diffusion test†	Agglutination test
1	PBS + adjuvant	5×10^7	4/6	4	5	0	4	5	6	5	6	0	0	1/16
		5×10^6	1/6	1	1	0	1	1	2	2	4	0		
		5×10^5	0/6	0	0	0	0	1	3	1	4	0		
2	Formalized spores + adjuvant	5×10^7	5/6	5	3	1* (spleen)	3	5	6	5	6	0	0	1/32
		5×10^6	0/6	0	0	0	0	0	1	0	1	0		
		5×10^5	0/6	0	0	0	0	0	1	1	3	0		
3	Hyphal walls + adjuvant	5×10^7	3/6	2	1	0	2	3	5	3	5	0	1	1/128
		5×10^6	0/6	0	0	0	0	0	0	0	2	0		
		5×10^5	0/6	0	0	0	1	0	1	0	2	0		
4	Mycelial extract + adjuvant	5×10^7	4/6	3	2	0	2	4	4	4	4	0	1–2	1/512
		5×10^6	0/6	0	0	0	0	1	0	2	0	0		
		5×10^5	0/6	0	0	0	0	0	0	0	0	0		
5	Spores + hyphal walls + adjuvant	5×10^7	2/6	2	1	0	1	2	2	2	4	0	1	1/128
		5×10^6	1/6	0	1	0	0	1	1	1	2	0		
		5×10^5	1/6	1	0	0	0	1	2	1	2	0		
6	Spores + hyphal walls + mycelial extract + adjuvant	5×10^7	2/6	2	1	1 (spleen)	1	2	3	3	2	0	1	1/256
		5×10^6	2/6	2	2	0	1	2	3	2	5	0		
		5×10^5	1/6	1	0	0	0	1	1	0	2	0		

* Associated with germinating spores; possible post-mortem development.

† Number of l.p.cs given by pooled sera on diffusion against *A. ramosa* mycelial extract.

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of antigenicity of the vaccines used, as precipitating and agglutinating antibodies were produced in response to these. Thus the preparations containing intracellular mycelial antigens, either alone or in combination with spores, hyphal walls or both materials, evoked precipitating antibodies on injection into mice. A similar but more intense response was produced in rabbits. On diffusion against *A. ramosa* mycelial extract the rabbit antisera produced at least 6 line pattern components (l.p.cs) whereas the mouse sera produced only 1 or 2 l.p.cs (Pl. 2, fig. 1).

The unvaccinated control animals or the mice injected with formalin-inactivated spores did not develop detectable antibodies to mycelial antigens. The sera of the mice vaccinated with preparations containing hyphal walls produced a single l.p.c. close to the antigen well when diffused against mycelial extract.

As indicated in Table 1, the pooled serum from unvaccinated animals or animals vaccinated with inactivated spores gave a low titre in latex agglutination tests. The titre of serum from the animals receiving hyphal preparations was somewhat higher but the highest titre was given by pooled serum from mice receiving mycelial extracts. Nevertheless it was quite clear that there was no relation between detectable antibody response and resistance to challenge.

Intradermal tests on animals vaccinated according to the various schedules failed to produce conclusive evidence of a greater frequency of delayed hypersensitivity to mycelial antigens in any of these animals than in unvaccinated control animals.

Most of the mice tested showed some infiltration by polymorphonuclear leucocytes at the injection site. This probably indicated a non-specific response to irritant material in the test antigen (Pl. 2, fig. 2).

Subcutaneous injection of germinating *A. ramosa* spores produced lesions containing fungal hyphae surrounded by extensive infiltrations of polymorphonuclear leucocytes and smaller number of macrophages. Multinucleate giant cells were not seen in histological sections prepared from tissues injected up to 10 days previously. The appearances of the fungal hyphae in these reactions were typical of those seen in acute phycomycotic lesions. These lesions usually resolved completely in 2–3 weeks (Pl. 2, figs. 3, 4).

Assessment of immunity to A. ramosa *in mice previously exposed to sub-lethal doses of viable spores*

Most animals given intravenous doses of *ca.* 5×10^4 viable *A. ramosa* spores survived without obvious ill effect. A small proportion, $< 10\%$ of those inoculated, developed acute cerebral phycomycosis and died.

On challenge of the survivors with intravenous doses of 5×10^6 spores no deaths resulted. With intravenous challenge doses of 5×10^7 spores *ca.* 20% of the inoculated animals developed acute lethal infection. As seen from Table 2 the death rate in the vaccinated group was significantly lower than in the unvaccinated control group ($P < 0{\cdot}01$, χ^2 test). In those animals of either group dying of acute infection, typical cerebral lesions were found at post-mortem examination.

The results of intracerebral challenge of mice vaccinated with sublethal doses of spores confirmed the considerable resistance of these animals to *A. ramosa*.

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Table 2. *The effect of previous exposure to viable* A. ramosa *spores on the susceptibility of mice to subsequent challenge*

Vaccine	Challenge dose of *A. ramosa* spores	Route of challenge	Number of deaths/number inoculated	Number of mice with fungal hyphae in the tissues of:			Number of mice yielding *A. ramosa* isolates on culture of the tissues of:						
				Brain	Kidney	Other organs	Brain	Kidney	Liver	Lung	Spleen	Bone marrow	Other organs
None	5×10^7	Intravenous	5/6	5	5	0	5	5	5	5	5	0	ND
	5×10^6		3/8	2	3	0	2	3	3	3	3	0	ND
	5×10^5		1/6	1	0	0	1	1	1	1	1	0	ND
5×10^4 viable *A. ramosa* spores	5×10^7	Intravenous	2/6	2	2	0	1	2	2	2	2	0	ND
	5×10^6		0/10	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	5×10^5		0/6	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
None	125	Intracerebral	7/10	7	1	0	6	1	0	0	0	0	ND
5×10^4 viable *A. ramosa* spores	125	Intracerebral	0/12	0	0	2*	0	0	0	0	0	0	2*

ND, Not done.
* Subcutaneous tissue. These non-lethal local lesions did not become apparent until *ca.* 6 months after inoculation.

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Whereas intracerebral injection of spore doses down to the level of single viable units produced lethal infection in unvaccinated mice, doses of 125 viable spores failed to produce acute infection in the vaccinated mice. Observation of the intracerebrally challenged animals over the succeeding 6 months failed to reveal any evidence of progressive chronic infection except in two mice which developed subcutaneous granulomatous lesions *ca.* 5 months after inoculation. The histological and mycological characteristics of these lesions were essentially similar to those described by Eades & Corbel (1975).

Precipitating antibodies to mycelial antigens were irregularly detected in the sera of mice vaccinated with viable *A. ramosa* spores. Sera from these animals gave reciprocal titres in latex agglutination tests ranging from 1/16 to 1/256.

All of the animals tested gave evidence of delayed hypersensitivity to intradermal injections of mycelial extract. Histological examination of the injection sites 24 hr. after inoculation showed infiltrations of mononuclear cells, predominantly of macrophage type, superimposed upon the low-grade non-specific polymorphonuclear response (Pl. 3, figs. 1, 2).

Subcutaneous injection of germinating *A. ramosa* spores provoked an intense cellular reaction. In samples taken between 24 and 48 hr. after injection, the cellular infiltrations consisted largely of polymorphonuclear leucocytes with smaller numbers of macrophages and lymphocytes (Pl. 3, fig. 3). Samples taken at a later stage in the evolution of the cellular reaction showed a more typically granulomatous response. Thus in specimens taken at 10 more days after injection, macrophages and epithelioid cells predominated in the cellular reaction (Pl. 3, fig. 4). Focal accumulations of plasma cells were also present (Pl. 4, fig. 1). The most characteristic feature of the cellular reaction at this stage was the presence of Langhans type multinucleate giant cells. These were usually immediately adjacent to fungal elements and in some cases hyphae were visible in vacuoles within the cytoplasm of these cells (Pl. 4, figs. 2, 3).

DISCUSSION

These results showed that vaccination with non-viable *A. ramosa* spores or mycelial components did not induce any significant degree of protective immunity to the fungus. This failure was clearly not attributable to lack of antigenicity of the vaccine preparations used as some of these at least, consistently induced detectable antibody responses to *A. ramosa* antigens. Nevertheless it is possible that these vaccines were deficient in labile antigens essential for the stimulation of protective immunity to the fungus. This explanation is speculative, however, as the nature of the components responsible for provoking protective immunity to mucoraceous fungi has yet to be defined.

An alternative explanation, consistent with the experimental evidence, is that the immunization procedures used for vaccination with non-viable preparations failed to induce the type of cellular response required for protection. Thus, although the procedures used evoked circulating antibodies to some of the antigens, they were not consistently effective in evoking delayed hypersensitivity to these. In

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view of the relation between delayed hypersensitivity and lymphocyte-mediated macrophage activation as manifestations of the cell-mediated immune response, this suggests that these vaccination procedures failed to achieve adequate priming of the lymphocyte population responsible for determining protective immunity.

The histological characteristics of the cellular reactions of immune animals vaccinated with viable spores, to both the soluble antigens and viable units of *A. ramosa,* strongly suggested that immunity to this organism was largely a cell-mediated process.

Thus the observation of hyphae within the cytoplasm of multinucleate giant cells in granulomata provided direct evidence of the role of activated macrophages in controlling the *in vivo* development of fungal mycelium. Normal macrophages are evidently capable of ingesting and preventing the germination of *A. ramosa* spores (Smith & Jones, 1973). However, the large size of fungal hyphae would prevent ingestion of these by single cells. This problem may be circumvented by the production of multinucleate giant cells formed by the fusion of macrophages Mariano & Spector, 1974). As multinucleate cells were not seen in acute lesions nor in the cellular reactions to *A. ramosa* produced by non-immune animals, it would appear that they fulfil a central role in the response of the immune animal to developing fungal mycelium.

It was notable that whereas immunized mice showed a considerable resistance to acute infection by *A. ramosa* spores, they were also more liable to develop persistent local granulomatous lesions than unimmunized animals. This may have been related to tissue damage resulting from hypersensitivity to the fungal antigens in the immune animals. On the other hand, it may have resulted from an inhibitory effect of precipitating antibodies on the breakdown of fungal antigens by macrophages. Such an effect has been observed in granulomatous infections (Cohn, 1963; Spector, Reichhold & Ryan, 1970). It may have been significant in this respect that both of the mice which developed subcutaneous granulomatous lesions *ca.* 6 months after serial intravenous and intracerebral inoculation of *A. ramosa* spores, had high titres of precipitating antibodies in their sera.

The reasons for the efficacy of sublethal doses of viable spores in stimulating immunity to *A. ramosa,* in contrast with the poor results obtained with non-viable preparations, are not known. It is possible, however, that inoculation with viable spores resulted in abortive infection with the production of protective antigens which were not produced *in vitro.*

On the basis of these results it may be concluded that specific immunity acquired after exposure to viable *A. ramosa* spores results in a substantial increase in the resistance of mice to acute infection by this fungus. This process probably also accounts for the increased resistance to phycomycotic infection developed naturally by older mice.

There seems little doubt that these animals acquire specific immunity to *A. ramosa* as a result of environmental exposure to this or to some antigenically related organisms. A similar response probably occurs in relation to other opportunistic fungi and was suggested by Smith (1972) as an explanation for the irregular susceptibility of mice to *Aspergillus fumigatus.*

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The acquisition of specific immunity would also explain the increased resistance of older mice to challenge by the intracerebral route. Thus it is well established that animals show little primary immune response to antigenic material implanted in the brain although they mount effective secondary responses if previously exposed to the antigen by another route (Medawar, 1948).

It may be concluded that the processes involved in the resistance of mice to *A. ramosa* are not fundamentally different from those implicated in immunity to other groups of micro-organisms.

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EXPLANATION OF PLATES

Plate 1

Fig. 1. Section through lung of a mouse injected intravenously with *A. ramosa* spores. PAS-positive fungal spores are visible in the interalveolar septum. PAS-light green, × 1000.

Fig. 2. Section through the brain of a mouse injected intravenously with *A. ramosa* spores. A typical phycomycotic lesion consisting of degenerating nervous tissue extensively infiltrated by predominantly mononuclear cells is visible near the IIIrd ventricle. Fungal mycelium is not visible in this preparation. Haematoxylin & eosin, × 250.

Fig. 3. A section of tissue similar to that shown in Fig. 2 but stained by the PAS method. Typical mucoraceous fungal hyphae are visible in the area corresponding to the lesion. PAS-light green, × 250.

Fig. 4. Section through kidney of a mouse showing acute phycomycotic lesions resulting from *A. ramosa* infection. The lesions are confined to the renal medulla and pelvis and consist of infiltrations of mononuclear and polymorphonuclear cells surrounding developing mycelium. The mycelium has remained unstained. Haematoxylin & eosin, × 250.

Plate 2

Fig. 1. Immunodiffusion of rabbit antiserum to *Absidia ramosa* mycelial antigens (aAR), murine antisera to inactivated spores (aSp), hyphal walls (aH), mycelial extract (aM) and a mixture of inactivated spores, hyphal walls and mycelial extract (aSpHM) and unvaccinated mouse serum (NMS) against *A. ramosa* mycelial extract (AR). Numerous l.p.cs are visible in the reaction between the rabbit antiserum and the antigen. From 1 to 2 l.p.cs are visible in the reactions between aH, aM, aSpHM and the antigen. No reaction is visible between the antigen and aSp or NMS.

Fig. 2. Section through a foot pad of a mouse previously vaccinated with a mixture of inactivated *A. ramosa* spores, hyphal walls and mycelial antigens. The specimen was taken 24 hr. after injection of *A. ramosa* antigens. A cellular reaction consisting mainly of infiltrations of polymorphonuclear leucocytes is apparent in the dermal and subcutaneous tissue. Haematoxylin & eosin, × 250.

Fig. 3. Section through a footpad of the mouse shown in Fig. 2 above, taken 24 hr. after local injection of saline. There is no evidence of any cellular reaction. Haematoxylin & eosin, × 250.

Fig. 4. Section through the skin and sub-cutaneous tissue of a mouse similar to that tested in Figs. 2 and 3, 2 days after local injection of germinating *A. ramosa* spores. An intense cellular reaction involving infiltrations of numerous polymorphonuclear leucocytes is evident in the subcutaneous tissue. Haematoxylin & eosin, × 250.

Plate 3

Fig. 1. Section through a footpad of a mouse vaccinated with a sublethal dose of viable *A. ramosa* spores, taken 24 hr. after local injection of *A. ramosa* mycelial antigens. A cellular reaction is evident in which infiltrations of mononuclear cells predominate. Haematoxylin & eosin, × 250.

Fig. 2. Section through a footpad of the mouse shown in fig. 1 taken 24 hr. after local injection of saline. There is no evidence of any cellular reaction. Haematoxylin & eosin, × 250.

Fig. 3. Section through the skin and subcutaneous tissue of a mouse similar to that shown in figs. 1 and 2. The sample was taken 2 days after local injection of germinating *A. ramosa* spores. A cellular infiltration of the subcutaneous tissue is apparent in which polymorphonuclear leucocytes predominate. Haematoxylin & eosin, × 250.

Fig. 4. Section through the skin and sub-cutaneous tissue of a mouse similar to that shown in figs. 1, 2 and 3. The sample was taken 14 days after local injection of germinating *A. ramosa* spores. Although infiltrations of polymorphonuclear leucocytes are still apparent in the cellular reaction, this has assumed a granulomatous appearance and many macrophages, epitheloid cells and multinucleate giant cells are visible. Haematoxylin & eosin, × 250.

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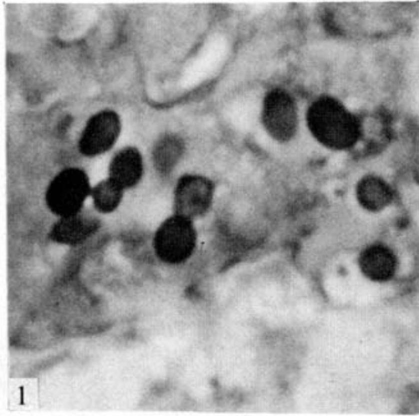

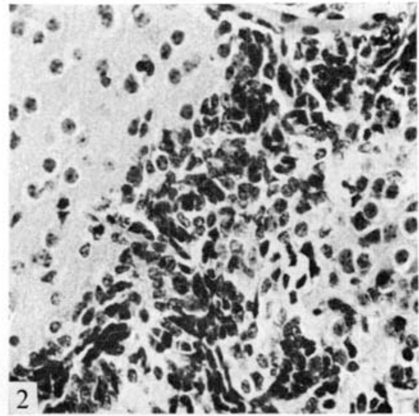

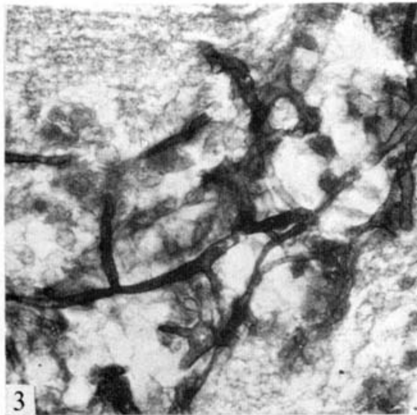

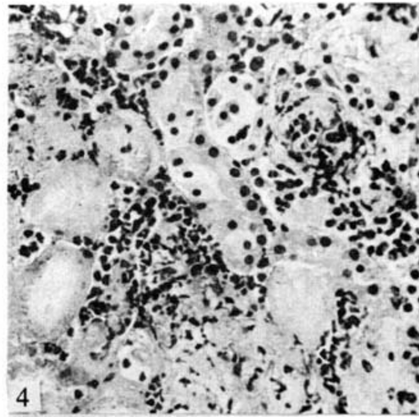

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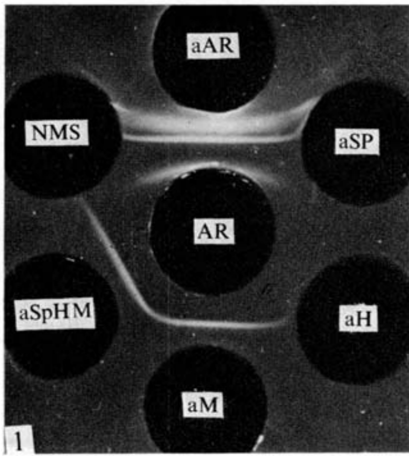

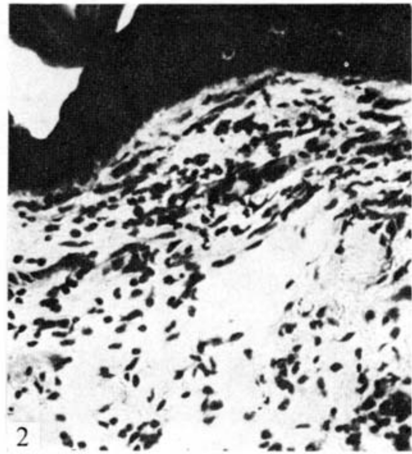

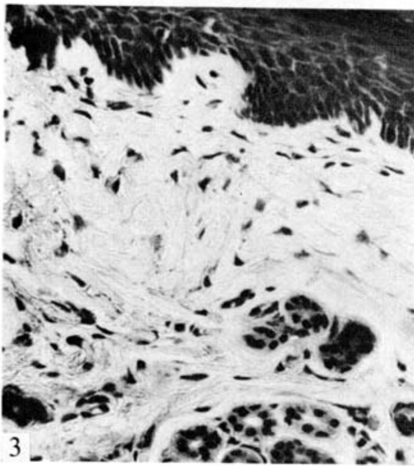

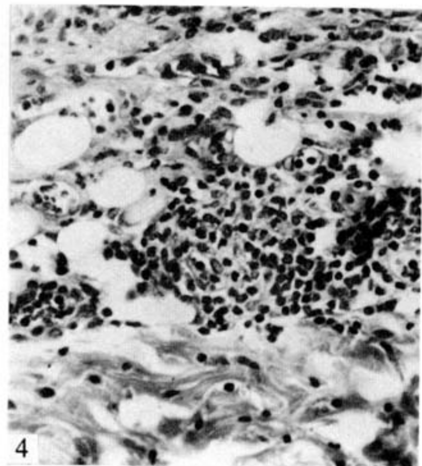

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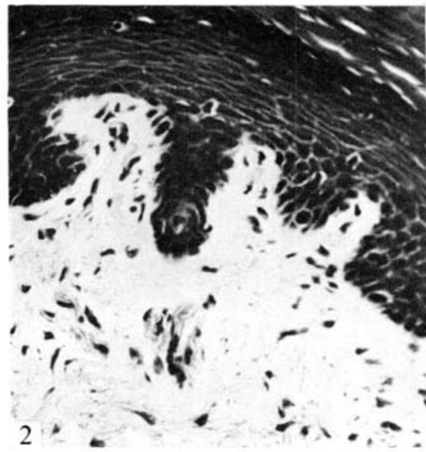

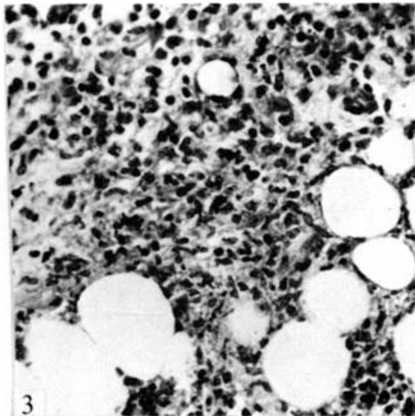

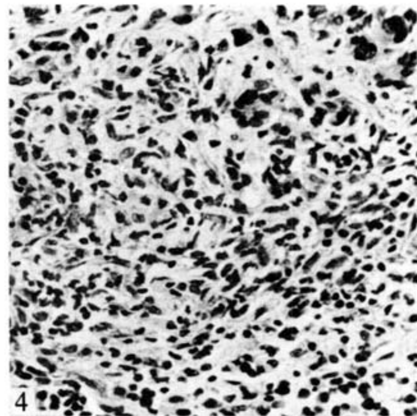

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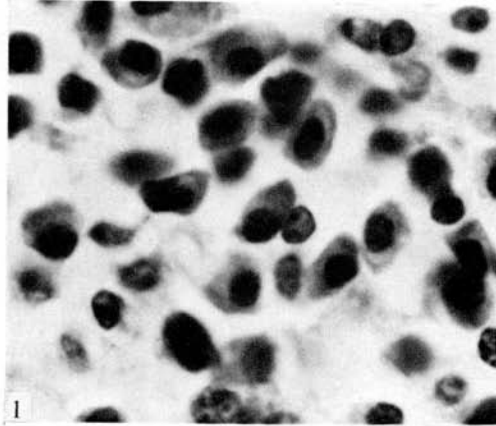

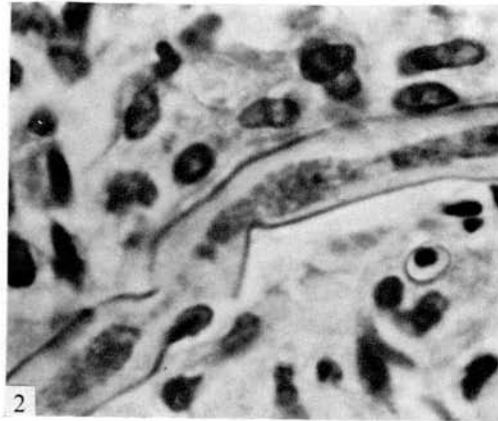

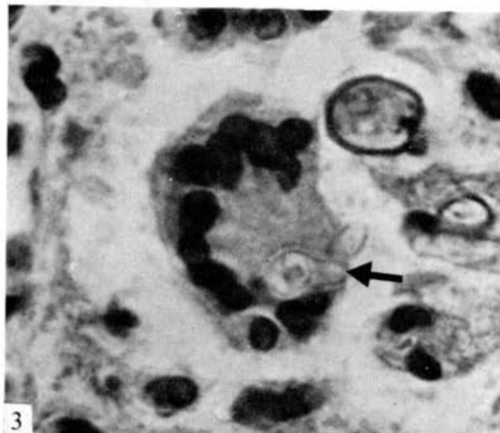

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PLATE 4

Fig. 1. Focal accumulation of plasma cells in a subcutaneous granuloma produced in response to local injection of *A. ramosa* spores in a mouse previously immunized with viable spores. Haematoxylin & eosin, × 1000.

Fig. 2. Section through a subcutaneous granuloma similar to that shown in fig. 1 above. A length of mucoraceous hypha is visible which is in the process of being surrounded by macrophages. Haematoxylin & eosin, × 1000.

Fig. 3. Section through a subcutaneous granuloma similar to that shown in figs. 1 and 2. This shows a multinucleate giant cell in the process of phagocytosing a length of fungal hypha. Haematoxylin & eosin, × 1000.

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