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Conditional Virulence of a *p*-Aminobenzoic Acid-Requiring Mutant of *Aspergillus fumigatus*

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The induced auxotrophy for *p*-aminobenzoic acid (PABA) resulted in a complete loss of virulence of *Aspergillus fumigatus* for normal as well as cortisone-treated mice. The PABA-requiring mutant of *A. fumigatus* survived in vivo for 4 to 7 days without causing any infection. However, it showed conditional virulence in animals receiving PABA in very small quantities. Repeated inoculations of the viable spores of the avirulent mutant strain gave favorable results in building immunity against intravenous challenge of the virulent strain. The immunogenicity of the PABA-requiring mutant was comparable with that of a wild strain of the fungus in agar gel double-diffusion tests using clinical and hyperimmune sera and in skin tests on patients with allergic bronchopulmonary aspergillosis.

The initiation of an infectious disease process depends inter alia on a favorable nutritional environment for the in vivo metabolism and proliferation of the causal organism. Studies on induced auxotrophs of bacterial pathogens of man, other animals, and plants have shown that the loss of biosynthetic ability for purines, aspartic acid, arginine, methionine, *p*-aminobenzoic acid (PABA), or some other metabolites can have an attenuating effect on their virulence (4, 7, 8, 13). Further, the virulence of some auxotrophs has been shown to be conditional on the in vivo availability of the required growth factor, thus separating the "nutritional" from the "inhibitory" environment that a pathogen encounters in its host (7). Buxton (5) and Boone et al. (3) have studied the effect of induced mutations on the pathogenicity of two plant pathogenic fungi, namely, *Fusarium oxysporum* and *Venturia inaequalis*, respectively. From among the fungi pathogenic to man and other animals, the influence of auxotrophy on virulence to experimental animals has been studied for *Coccidioides immitis* (14, 23), *Candida albicans* (11), and *Aspergillus nidulans* (17). Earlier, with a view to demonstrating the occurrence of parasexual genetic recombination, Strømnaes and Garber (21) had successfully evolved a number of nutritionally deficient mutants in *Aspergillus fumigatus*, but so far no attempt has been made to study the pathogenicity of auxotrophs in this fungus.

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A. fumigatus is an important opportunistic fungal pathogen causing a protean disease in man and other animals. The present paper reports the complete loss of virulence accompanying an induced auxotrophy for PABA in a human isolate of *A. fumigatus*. The mutant is of interest since its virulence is conditionally restored by administering the required growth factor to experimental mice and is apparently fully immunogenic in clinical tests.

MATERIALS AND METHODS

Organisms. *A. fumigatus* strain 1297, isolated locally from the lung biopsy of a human case of aspergilloma, was designated as the wild type (19). All mutants were derived from this strain by chemical mutagenesis. In addition, *A. fumigatus* strain SP285, isolated from sputum, was used for the preparation of antigens required in the routine immunological and serological diagnostic procedures.

Culture media. The minimal and complete media of Donkersloot and Mateles (6) were used throughout the study, and cultures were incubated at 37 C for 4 to 5 days to obtain good yields of conidia. The fungal stocks were maintained in soil cultures in the cold, with periodic subculturing on minimal or complete medium agar slants to raise spore inoculum for experimental work.

Metagenesis. Conidia from fresh complete medium agar slants were harvested in 0.5% Tween 80. The suspensions were diluted in minimal medium to obtain 10^8 spores/ml. The conidial suspensions were treated with a mutagenic agent, *N*-methyl-*N'*-*N*-nitrosoguanidine (Aldrich Chemical Co. Inc., Milwaukee, Wis.) at a concentration of 0.5 mg/ml for 1 h (1). From the treated suspensions master plates were prepared after suitable dilutions in complete me-

dium containing deoxycholate. The auxotrophic mutants were isolated by the replica plating technique (12, 18). After thorough verification on minimal and supplemented media, two auxotrophs were found to have an absolute requirement for PABA (paba-1 and paba-2) and one required ammonium nitrogen (am-1), whereas one isolate (1297t) was prototrophic like the wild type (1297), manifesting no alteration in its nutritional or morphological characters.

Pathogenicity. Six- to 8-week-old male white mice, bred in the animal house of the Vallabhbhai Patel Chest Institute, were used for pathogenicity studies. The inoculum consisted of conidial suspensions prepared from freshly grown slants. The conidia were harvested in a small quantity of 0.5% Tween 80 and further diluted with sterile normal saline to reach the desired concentration of 5×10^6 spores/ml. Each animal was challenged with 10^6 spores injected in to the tail vein, unless stated otherwise. Mortalities were observed for a period of 3 weeks. Portions of visceral organs of the animals autopsied or sacrificed were cultured on complete medium and also fixed in 10% formalin for histopathology. The pathogenicity studies included virulence of the wild-type and mutant strains (1297, paba-1, paba-2, am-1 and 1297t), in vivo survival of the paba-1 mutant, and the effect of administration of cortisone and PABA on its pathogenicity.

Administration of cortisone and PABA. Cortisone (Roussel) was injected intramuscularly (i.m.) into the hind leg of mice, each animal receiving a single dose of 5 mg (i.e., 250 mg/kg of body weight) just before challenge with the test strain of *A. fumigatus*. PABA was administered by two routes. To one batch of mice it was injected i.m. at a daily dose of 1 mg (i.e., 50 mg/kg of body weight) to each animal. To the second batch of mice it was added in the drinking water at a concentration of 1 mg/ml, and the animals were allowed to drink ad libitum with a daily change of fresh solution.

Immunization. The following two schedules of immunization of white mice with the avirulent strain paba-1 were used. (i) Three intravenous (i.v.) inoculations of 10^6 viable spores of the paba-1 mutant were given to each mouse at weekly intervals before challenging them at the end of week 4 with the virulent strain 1297, and (ii) six weekly i.m. inoculations of 10^7 spores of the paba-1 strain preceded the challenge at the end of 7 weeks with the virulent strain. The control groups were not immunized and were challenged either with paba-1 or strain 1297 only.

Antigens. The antigenic properties of the paba-1 mutant of *A. fumigatus* were compared with the wild type and another strain, SP285, routinely used in this laboratory for diagnostic work in patients with allergic aspergillosis and aspergilloma. Antigens were prepared by growing the fungus in glucose-asparagine medium (20) for 4 weeks at 27 ± 1 C. After Seitz filtration, the culture filtrates were dialyzed against running water for 24 h and finally against distilled water containing 100 μg of merthiolate per ml as preservative.

Skin tests. Intracutaneous tests were performed in selected patients by injecting 0.02 ml of the antigens. The reactions were read after 15 min and between 4 to 8 h.

Serology. The dialyzed filtrates were concentrated 10- to 20-fold for the immunodiffusion tests. Hyperimmune sera against paba-1, 1297, and SP285 strains were raised in rabbits. Each animal was given three weekly i.m. injections of a 1-ml suspension of dried and defatted mycelium along with 1 ml of Freund adjuvant. The animals were bled at the end of 6 weeks for the collection of antisera (2). The double-diffusion tests were carried out in 50-mm agar plates, using McIlvaine citrate buffer (pH 7.3), by the methods of Proctor (16). Each plate had six peripheral wells of 6 mm in diameter with a 6-mm edge-to-edge distance from the central well, also of the same diameter. The precipitin bands were allowed to develop for 3 to 4 days at 30 C. The plates were washed in normal saline, and the bands were stained with amido black with final washing in 2% acetic acid.

RESULTS

The pathogenicity tests for the wild type and four mutant strains of *A. fumigatus* revealed that two of the auxotrophic mutants, namely, paba-1 and paba-2, both having absolute growth requirement for PABA, were completely avirulent to mice. This was apparent from the fact that neither mortality nor morbidity was recorded for these two strains (Table 1). The ammonium nitrogen-dependent mutant am-1, on the other hand, retained its virulence, causing 50% mortality. Strain 1297t, which apparently had undergone no change in its physiological or morphological characteristics due to mutagenic treatment, killed 80% of the animals as compared to the 90% mortality recorded for the wild type (1297). Histopathological study mostly showed infection in the kidneys, except for two animals in which the heart was also involved. The fungal lesions consisted of ab-

TABLE 1. *Pathogenicity of auxotrophic mutants of A. fumigatus*

A. fumigatus strain inoculated[a]		Mortality and morbidity	
No.	Nutritional deficiency	No. of deaths	No. showing histopathological lesions
paba-1	PABA	0	0
paba-2	PABA	0	0
am-1	Ammonium nitrogen	5	5
1297t	None (NTG treated)	8	8
1297	None (wild type)	9	10

[a] Each strain was tested in a batch of 10 white mice by injecting 10^6 spores/animal i.v. NTG, *N*-methyl-*N'*-*N*-nitrosoguanidine.

scesses having a central mass of branching hyphae surrounded by inflammatory cells, chiefly polymorphs, and frequently showing some necrosis.

The in vivo survival of the avirulent PABA-requiring mutant paba-1 was followed in a batch of 20 mice after i.v. inoculation of one million spores per animal. Two animals were sacrificed every day for the first 7 days and then at weekly intervals up to 28 days. The cultures of their internal organs on PABA-supplemented medium were positive for about 4 to 7 days, after which the mutant was not recoverable from the inoculated animals (Table 2). In another experiment an attempt was made to enhance the susceptibility of experimental mice by cortisone treatment, but the paba-1 mutant failed to infect the animals thus treated. The organs of the challenged animals were invariably free from any fungal lesions (Table 3), thus further suggesting the avirulent nature of the auxotroph.

In view of its absolute growth requirement for PABA, the effect of the intake of this growth factor by mice on the virulence of the paba-1 strain of *A. fumigatus* was studied. In the two batches of mice receiving PABA orally or i.m. and challenged with the avirulent paba-1 strain, 19 (95%) and 7 (35%) mortalities, respectively, were recorded during the 2-week observation period (Table 4). In the two control groups, that is, the mice receiving either spore inoculum or PABA alone, neither mortality nor morbidity was recorded. It was noteworthy that the restoration of virulence of the otherwise nonpathogenic mutant was almost complete for the batch of mice put on oral intake of PABA.

In view of the in vivo survival of the paba-1 mutant for only a limited period after i.v. inoculation in mice, the possibility of effectively

TABLE 2. *In vivo survival of the paba-1 mutant of A. fumigatus*

Organ cultured	Recovery of *A. fumigatus* in culture (days after inoculation)[a]									
	1	2	3	4	5	6	7	14	21	28
Kidneys	+	+	+	+	+	+	−	−	−	−
Liver	+	+	+	+	+	+	−	−	−	−
Spleen	+	+	+	+	±	+	±	−	−	−
Heart	+	+	+	+	+	±	±	−	−	−
Lungs	+	+	+	+	±	+	−	−	−	−
Brain	+	+	+	+	−	−	−	−	−	−

[a] Two mice were sacrificed on each day indicated after i.v. inoculation of 10^6 spores/animal. +, Positive cultures from both animals; ±, positive cultures from only one animal; −, negative cultures. None of the sacrificed animals showed histopathological lesions.

TABLE 3. *Pathogenicity of the paba-1 mutant of A. fumigatus in normal and cortisone-treated mice.*

A. fumigatus strains inoculated[a]	Mortality (in batches of 10 mice each)	
	Normal	Cortisone treated[b]
paba-1	0	0
1297 (wild type)	8	10
Control (not challenged)	0	0

[a] Each animal was given an i.v. dose of 10^6 spores.
[b] Each animal received 5 mg of cortisone i.m. prior to challenge.

immunizing the animals with the avirulent strain was explored. The mortality and morbidity observed for a period of 15 days after the challenge were greatly reduced in the immunized batches as compared with the control group, which registered 18 dead in a batch of 20 mice (Table 5). In the batch of animals immunized by the i.m. route, the mortalities were minimum (only 3 dead out of 20 mice), not exceeding those in the avirulent control, and there was no histopathological evidence of infection in any of the sacrificed animals. This showed that the i.m. route of immunization was quite effective in building immunity against the virulent strain, whereas the i.v. immunization route was only partially so.

To assess the immunogenic properties of the paba-1 mutant, its dialyzed culture filtrate was used in intracutaneous tests. These were carried out in patients known to be suffering from allergic bronchopulmonary aspergillosis. All five patients tested responded with dual skin reaction; that is, the immediate type I wheal and flare was characteristically followed at 4 to 8 h by an edematous swelling (type III) at the site of injection, measuring over 50 mm in diameter. The potency of aspergillin from the mutant was comparable to the one routinely used (*A. fumigatus* strain SP285) in the diagnostic work in this laboratory. Likewise, the serum samples derived from four patients with allergic bronchopulmonary aspergillosis and one with aspergilloma yielded one to three precipitin bands against the paba-1 antigen in the double-diffusion test. The serum of the aspergilloma patient showed one band of nonidentity and two of identity with those of patients with allergic bronchopulmonary aspergillosis (Fig. 1).

The hyperimmune serum of strain SP285 revealed four precipitin bands when tested against the culture filtrates of paba-1, and all of these bands were common with the homologous antigen (Fig. 2). The wild type, 1297, also yielded similar results, thus demonstrating ap-

TABLE 4. *Effect of administration of PABA on the virulence of the paba-1 mutant of A. fumigatus to mice*

A. fumigatus strain inoculated[a]	Administration of PABA		Mortality and morbidity (in batches of 20 mice each)		
	i.m. (1 mg/day)	In drinking water (1 mg/ml)	No. of deaths	No. showing histopathological lesions	No. yielding *A. fumigatus* in culture
paba-1	−	+	19 (95%)	16	12
paba-1	+	−	7 (35%)	13	12
paba-1	−	−	0	0	0
Control (not challenged)	+	+	0	0	0

[a] Each mouse was given an i.v.dose of 10^6 spores.

TABLE 5. *Immunization of white mice with viable spores of the avirulent paba-1 mutant against i.v. challenge of virulent wild-type strain 1297 of A. fumigatus*

Route of immunization (with avirulent strain paba-1)	i.v. challenge with 10^6 spores of virulent strain 1297	Mortality (in batches of 20 mice each)	
		No. of deaths	No. showing histopathological lesions
i.v.[a]	+	10	10
i.m.[b]	+	3	0
Not immunized	+	18	18
Not immunized	Challenged only with avirulent strain	3	0

[a] Each animal received 10^6 viable spores at weekly intervals for 3 weeks prior to the challenge.
[b] Each animal received 10^7 viable spores at weekly intervals for 6 weeks prior to the challenge.

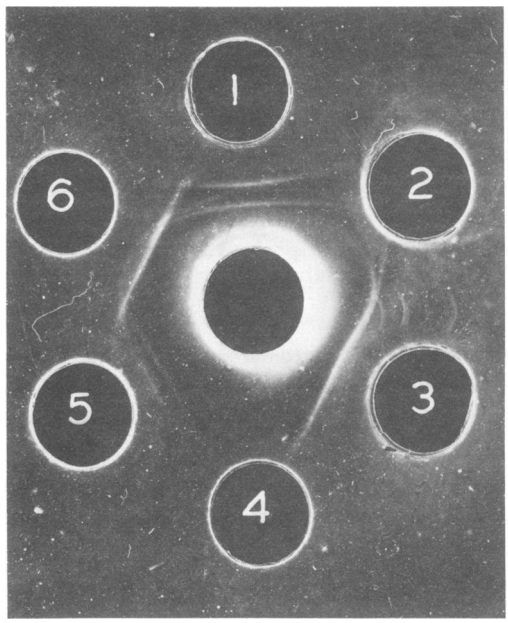

FIG. 1. *Agar gel double-diffusion test. The central well contained the antigen of A. fumigatus strain paba-1, and sera of four different patients with allergic bronchopulmonary aspergillosis were added to peripheral wells 1, 2, 4, and 5. Wells 3 and 6 had serum from an aspergilloma patient. Note the bands of complete, partial and nonidentity between various sera (see text).*

parently the unaltered antigenic properties of the paba-1 mutant.

DISCUSSION

The results of the pathogenicity tests for the PABA-requiring mutant of *A. fumigatus* indicate the causal relationship between PABA deficiency and avirulence. The evidence in favor of this conclusion is twofold. Firstly, of the four strains isolated after the mutagenic treatment of the virulent wild-type strain of the fungus, only two, both requiring PABA for growth, proved avirulent. The other two strains, one prototrophic and one requiring ammonium nitrogen, were pathogenic when inoculated i.v. into white mice (Table 1). Secondly, the pathogenicity of the paba-1 mutant could be conditionally restored if the animals were administered small amounts of PABA either orally or i.m. to ensure its in vivo availability to the pathogen (Table 4). Earlier, Walch and Kalvoda (22) observed that in *C. immitis*, a highly infectious fungus, PABA deficiency was invariably associated with avirulence or low virulence when inoculated intratesticularly or intranasally into white mice. Similar results have been reported for *A. nidulans*, in which the comparative pathogenicity of a variety of auxotrophs has been tested recently by Purnell (17). There are now considerable data available both for bacterial and fungal pathogens, which indicate that avirulence or decreased virulence attendant on deficiencies for a variety of nutrilites, such as purines, aspartic acid, arginine,

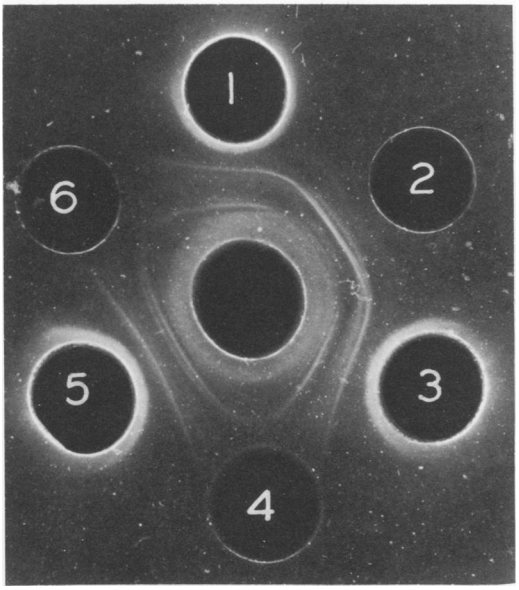

FIG. 2. *Agar gel double-diffusion test. The central well contained hyperimmune rabbit serum against A. fumigatus strain SP285, and antigen of strain paba-1 was added to peripheral wells 1, 3, and 5, the homologous antigen was added to well 2, and those of A. flavus and A. niger were added to wells 4 and 6, respectively. Note the bands of identity between the two strains of A. fumigatus and absence of visible bands against the other two species.*

methionine, cysteine, PABA, riboflavin, etc., may be reversed either by a back mutation to prototrophy or simply by injecting the nutrilites simultaneously with the inoculum (4, 5, 7–10). The virulence of such mutants, including in particular the PABA-requiring auxotrophs, is therefore directly related to the in vivo availability of essential metabolites or growth factors.

The conditionally virulent paba-1 mutant of *A. fumigatus* seems to be uniquely suited for controlled production of disease in experimental animals by manipulating the exogenous supply of the critical growth factor, PABA. This should provide a new approach to the study of pathogenesis in experimental aspergillosis. In addition, its utility as a safe material to handle in the production of antigenic preparations for serological and immunological diagnostic work is self-evident. The antigenicity of the PABA mutant of *A. fumigatus* was fully demonstrable in agar gel double-diffusion tests against both hyperimmune and clinical sera (Fig. 1 and 2). Moreover, skin tests with culture filtrates of the mutant elicited positive type I and type III hypersensitive responses (15) in patients with allergic bronchopulmonary aspergillosis. The PABA-deficient strain of *A. fumigatus* is apparently also capable of building a fair degree of immunity in white mice against i.v. challenge with the wild-type virulent strain, and more effectively so by the i.m. route. This suggests its possible usefulness as a source of live vaccine. It has been shown for *C. immitis* that subcutaneous inoculation of viable arthrospores of its avirulent diauxotrophs renders mice immune to a certain challenge dose of the virulent prototroph, and the degree of immunity in this infection is dependent on the production of primary disease, which resolves spontaneously (22). The inability of the paba-1 strain of *A. fumigatus* to revert to prototrophy precludes the risk of infection by the vaccine itself, provided PABA is withheld in feed. It may be borne in mind that genetic changes arising out of deletion, inversion, or translocation are among the least susceptible to spontaneous reversions. There is, however, no way of verifying in the imperfect fungus *A. fumigatus* whether any of these chromosomal aberrations is responsible for PABA deficiency in the mutant under discussion.

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