GENETIC AND MORPHOLOGICAL PROPERTIES OF UNDIFFERENTIATED AND INVASIVE VARIANTS OF *ASPERGILLUS NIDULANS*[1]

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FUNGI may provide excellent material for the study of abnormal growth and differentiation since they can be subjected to detailed morphological, histological, biochemical, and genetic analysis. The idea of using a microbial system as a model for investigating the basic mechanisms of neoplastic growth has been proposed on several occasions in the last two and a half decades. Stimulated by WARBURG's (1930) proposal that neoplastic cells are biochemical lesions of normal cells with enhanced glycolysis and impaired respiration, the first model probably stems from the work of STIER and CASTOR (1941) who isolated small yeast cells with these properties, i.e., the petites. GAUSE (1966) has written a comprehensive review of the microbial systems employed as models of cancer cells.

Over the years numerous morphological variants have been studied in Neurospora at the genetic and/or biochemical level (SRB 1963; JINKS 1964; BRODY and TATUM 1967 and GARNJOBST and TATUM 1967). In *Aspergillus nidulans*, ROPER (1958) isolated a group of mutants, mycelials, which superficially resemble the fluffy variants described in this paper. However, the mycelials, unlike the fluffies, were induced with acridine and are unstable. In addition, they are controlled by a different set of nuclear genes and are unable to infiltrate into an established and fully differentiated Aspergillus colony.

In general, there is a sharp zone of demarcation between two adjacent colonies of Aspergillus, growth apparently ceasing at this junction. Recently, DORN, MARTIN and PURNELL (1967) described two morphological variants (*flu2* and *3*) in Aspergillus which show delayed differentiation and were capable of invading into an established non-fluffy colony. The term "invasion" is used here to describe the series of events whereby a fluffy variant infiltrates and progressively covers the entire surface of a fully differentiated (non-fluffy) *Aspergillus nidulans* colony. As these variants might prove valuable for biochemical studies of the mechanisms controlling differentiation, a search for additional mutants was undertaken. The purpose of this paper is to describe the properties of the variants, especially morphological and genetical.

MATERIALS AND METHODS

Strains: The *Aspergillus nidulans* strains used in this study were from the stock collection

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held in the Department of Genetics, Glasgow University or were meiotic recombinants of these strains. For details of the strains, symbols and routine methods employed, reference should be made to PONTECORVO *et al.* (1953), PONTECORVO and KÄFER (1958), BARRATT, JOHNSON and OGATA (1965) and DORN (1967).

Media: Minimal medium (MM): $NaNO_3$, 6g; $MgSO_4 \cdot 7H_2O$, 0.52g; KCl, 0.52g; KH_2PO_4, 3g; glucose, 10g; Bacto agar (Difco), 1.3g; zinc, iron, manganese, copper and molybdate traces; 1 liter deionized water; pH adjusted to 6.5 with 4N NaOH before sterilization. Complete medium (CM): minimal medium plus Bacto peptone (Difco), 2g; vitamin-free casamino acids (Difco), 1.5g; yeast extract, 1.5g; adenine sulfate, grade B (Calbiochem), 0.1g; and 1 ml of vitamin solution; pH adjusted to 6.2 with 4N NaOH before sterilization. Vitamin solution: riboflavin, U.S.P. 10 mg; niacin, U.S.P. 10 mg; *p*-aminobenzoic acid, U.S.P. 5 mg; pyridoxine monohydrochloride, U.S.P. 5 mg; thiamine HCl, U.S.P. 5 mg; Ca-pantothenate; U.S.P. 5 mg; (—) biotin, U.S.P. 0.2 mg; choline chloride, 10 mg; ascorbic acid, U.S.P. 5 mg; folic acid, U.S.P. 5 mg, and 10 ml deionized water. Steam sterilization was carried out at 15 lbs. of pressure for 15 min.

Chemicals: Tetracycline hydrochloride, puromycin dihydrochloride, and hydroxy urea were purchased from Nutritional Biochemical Corporation; chloramphenicol, streptomycin sulfate, and all of the vitamins were obtained from Calbiochem. All other reagents were Baker analytical grade.

Genetic symbols: Visibles: *bl*–blue ascospores; *cha*–yellowish green conidia; *fw*–yellowish brown conidia; *w*–colorless conidia; *y*–yellow conidia; *yg*–mixture of yellow and green conidia on CM.

Resistant: *Acr*-acriflavin

Nutritional: *ad*–adenine; *an*–thiamine (aneurin); *arg*–arginine; *bi*–biotin; *lu*–leucine; *lys*–lysine; *ni*–nitrite; *nic*–nicotinic acid; *paba*-*p*-aminobenzoic acid; *phen*–phenylalanine; *pyro*–pyridoxine; *ribo*–riboflavin; *s*–sulfite; *thi*–thiazole.

Carbon source: *gal*–galactose

Master strains: MSD—a multiply-marked strain carrying at least one mutant locus on each of the eight linkage groups; genotype with semicolon separating the linkage groups: *su1ad20 y ad20*; *Acr1*; *phen2*; *pyro4*; *lys5*; *s3*; *nic8*; *ribo2*.
MSE–similar to MSD except for four marker changes; genotype: (*su1ad20*) *y*; *w2*; *gal1*; *pyro4*; *facA303*; *s3*; *nic8*; *ribo2*.

Miscellaneous: *su*–suppressor; *pal*–alkaline phosphataseless (pH 8.2); *pac*–acid phosphataseless (pH 4.8); *palc*–acid and alkaline phosphataseless.

Isolation of mutants: All of the fluffy variants were isolated from stock cultures which had been stored at 4 or 25°C for at least 6 months. In each case, the variant arose spontaneously as an undifferentiated, cotton-like and aerial hyphal mass growing over the surface of an established non-fluffy slant. Where possible, the strains were purified by single-spore plating. For those strains which failed to conidiate, they were purified by repeated transferring of hyphal tips. Although many stock cultures repeatedly yielded fluffy outgrowths, each variant described in this study arose from a genetically different strain.

One fluffy strain, MSE *flu7*, was selected for reversion studies. Conidial suspensions were exposed to ultraviolet light (2537 Å, Raymaster lamp, 8 w), giving approximately 5% survival and incubated on CM at 37°C. After 3 days the plates were visually inspected; those colonies which showed restoration of normal differentiation were purified by single-spore plating. To make sure that each revertant was of independent origin, only one mutant was taken from each irradiation and the conidia for each irradiation were harvested from an isolated colony of *flu7* which arose from a single conidium (conidia of *Aspergillus nidulans* are uninucleate).

Genetic analysis: Where feasible, location of the genetic loci was accomplished in the following manner: the mutant allele was first assigned to a particular linkage group by mitotic analysis and then located with respect to other markers in that group by meiotic analysis. The assignment of a mutant to a particular linkage group is made possible by the use of the parasexual cycle

(PONTECORVO 1956) and the technique of mitotic haploidization (PONTECORVO, TARR GLOOR and FORBES 1954; KÄFER 1958; FORBES 1959). The utilization of this technique is simplified by the availability of tester strains (symbols: MSD, MSE, and MSF) with markers on each of the eight linkage groups (MCCULLY and FORBES 1965) and the use of *p*-fluorophenylalanine, which facilitates the isolation of haploids from diploid strains (LHOAS 1961; MORPURGO 1961).

Random spore isolation from single cleistothecia was employed for the location of a mutant with respect to other markers in the same linkage group.

RESULTS

General properties: A total of nine fluffy variants has been chosen for analysis. Each arose spontaneously in a genetically distinct stock culture as an undifferentiated cotton-like mass of aerial hyphae. With the exception of *flu8*, the genetic composition of each isolate was identical to the non-fluffy strain from which it arose, thereby ruling out contamination by another fungus. Morphologically, all the fluffy strains fail to differentiate normally. Conidiation is delayed or completely absent and, in most cases, the strains are self-sterile. A few of the more severe forms are cross-sterile as well. Upon purification, each fluffy strain assumes a stable and characteristic morphology which is readily recognizable.

As was mentioned above, there is a zone of demarcation between two adjacent non-fluffy Aspergillus colonies. All of the fluffy strains, however, can infiltrate into and grow over the surface of an established and well differentiated colony (Figure 1).

In this laboratory, the occurrence of fluffy overgrowth in the stock cultures has reached epidemic levels. Approximately one hundred out of three hundred genetically distinct strains have yielded one or more fluffy outgrowths within the last two years. Many strains are especially prone to exhibit this phenomenon, i.e., *bi1* and *bi1; yg*. As the fluffy growth proceeds up a previously established stock culture tube, it becomes progressively more difficult to recover the original non-fluffy strain. This suggests that the fluffy mycelia not only grow over the surface of the parent strain but also enter into a heterocaryotic state with it and thereby cause the fluffy to spread ahead of the invading aerial hyphal mass.

Characterization of the fluffy variants: An attempt has been made to separate the fluffy strains into individual classes on the bases of morphology at 25° and 35°C, drug response, and genetic properties. With these criteria, it has been possible to place each variant into a distinct class. It is clearly recognized that this type of approach may not be completely adequate; yet the already noted differences will be helpful in planning future genetic and biochemical experiments.

The origin, morphological characteristics, and unusual features of each fluffy strain and four UV-induced revertants of *flu7* are listed in Table I. The general appearance of the individual fluffy strains when grown on CM agar plates is shown in Figures 2–8. Basically, three patterns of growth are evident: (1) abundant aerial hyphae (Figures 3a, 3b, 3c, 3d, and 6a); (2) flat, succulent growth with the mycelia arranged in a counterclockwise whorl or in concentric rings (Figures 4 and 5); and (3) matted growth with surface covered with a fine

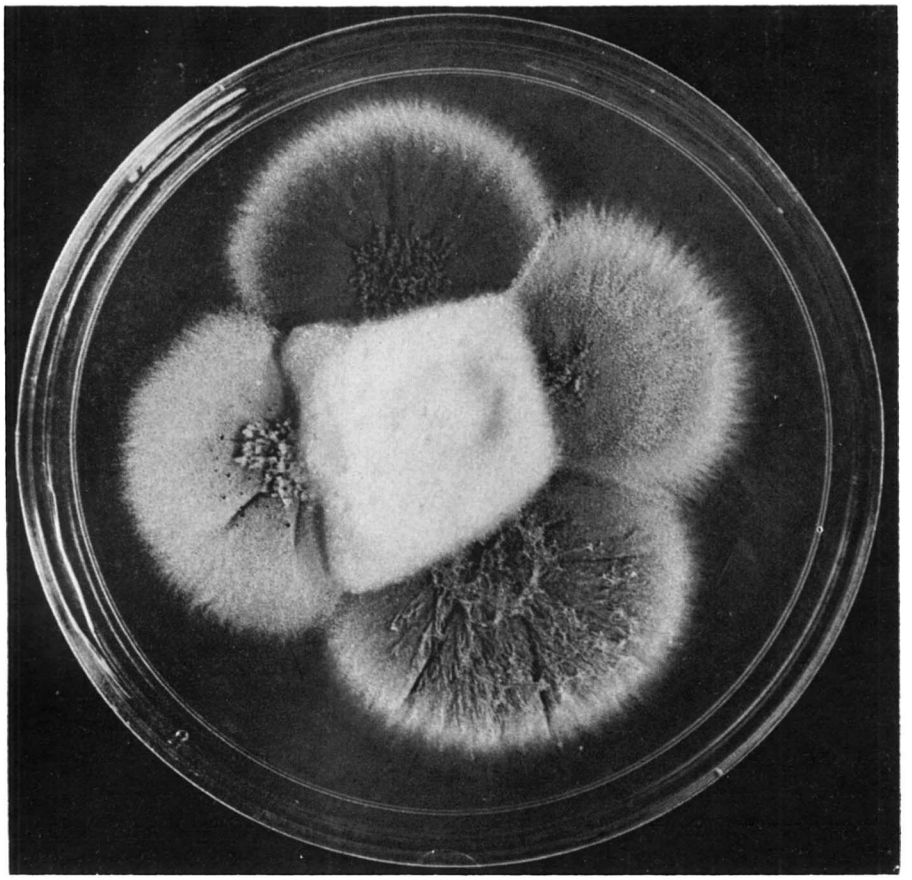

FIGURE 1.—Invasion of *flu13* into four non-fluffy strains. Center, *bi1*; *flu13*; top, *bi1*; left, MSE; right, *ad23*; *facA3*; *cha*; bottom, *bi1*; *yg*.

hair-like layer (Figures 7 and 8a). The time and pattern of conidiation is variable but conidiation is always delayed or absent and characteristic of the strain in question. *flu4* and *13* are self- and cross-sterile, while *flu1* and *11* show an unusually abundant number of self-cleitsothecia. *flu11* no longer expresses the *palA1* mutation and *flu8* and *12* are unusual in that they display temperature-sensitive properties (Figures 6a, 6b, 8a and 8b). At 25°C *flu8* possesses the characteristics common to all of the other fluffy mutants; however, at 37°C it grows poorly and releases a diffusible compound which inhibits the growth of other Aspergillus strains (Figure 9). This strain is also unusual in that it no longer has, phenotypically at least, the genetic markers of the parent strain, namely, *y*, *nic2 facA303 ribo5*. Although its hyphal structure and branching resembles that of the parent strain, the possibility that *flu8* is a contaminant fungus of unknown type has not completely been ruled out. The temperature-sensitive strain, *flu12*, has an essentially wild-type morphology at 37°C (Figure

TABLE 1

Description of the fluffy variants

Symbol	Origin: Treatment and strain	Description*	Isolated by
flu1	Spontaneous in (su1ad20) gal5 an1 lu bi1	Abundant undifferentiated aerial hyphae collapse by the 6th day, conidiation by 3rd day, hypersexual.	G. Dorn
flu2	Spontaneous in y; w2 thi4 ni3 ad3 bi1	Flat, succulent and counterclockwise whorled growth, very sparse conidiation.	G. Dorn
flu3	Spontaneous in ad23; facA3; cha	Abundant aerial hyphae; uniform conidiation by 5th day.	R. Holz
flu4	Spontaneous in bi1; yg	Abundant undifferentiated aerial hyphae which collapse centrally by 4th day, no conidiation, self and cross sterile.	W. Rivera
flu7	Spontaneous in MSE	Flat succulent base with tufts of mycelia grouped centrally and in concentric rings, moderate conidiation by 4th day.	G. Martin
su1 flu7; flu7	UV, MSE flu7	Similar to wild type, 1 day delay in conidiation; phenotypically palc.	G. Dorn
su2 flu7; flu7	UV, MSE flu7	Similar to wild type, 1 day delay in conidiation; phenotypically pac.	G. Dorn
su4 flu7; flu7	UV, MSE flu7	Wild-type morphology; phenotypically fac$^+$.	G. Coulon
flu7; rv5 (revertant)	UV, MSE flu7	Wild-type morphology.	G. Coulon
flu8	Spontaneous in y; nic2 facA303 ribo5	37°C.: very limited growth, no conidiation 25°C.: flat surface covered with hairy aerial hyphae, no conidiation, phenotypically nic+, fac+ and ribo+, self and cross sterile.	G. Dorn
flu11	Spontaneous in y; palA1 gal1 phen2	Matted with radiating troughs, covered with hairy aerial hyphae, uniform conidiation by 6th day, hypersexual, phenotypically pal+.	W. Rivera
flu12 (temperature sensitive)	Spontaneous in y; nic8 mal1 palF15	37°C.: basically wild-type morphology, normal conidiation by 3rd day. 25°C.: abundance of aerial hyphae, no conidiation.	G. Dorn
flu13	Spontaneous in bi1	Abundant central aerial hyphae with succulent periphery, very sparse central conidiation by 6th day, self and cross sterile.	G. Dorn

* Unless noted otherwise, the fluffy variants have similar properties at both 37°C. and 25°C.

6b) but produces an abundance of undifferentiated aerial hyphae at 25°C (Figure 6a).

Classification of the MSE flu revertants: MSE *flu7* was selected for reversion studies by UV-irradiation. A total of four revertants of independent origin (see MATERIALS *and* METHODS) have been isolated from approximately, 10,000 surviving colonies. To date, no spontaneous revertant has been detected. They are all morphologically similar to the wild-type parent strain, MSE (Table 1). However,

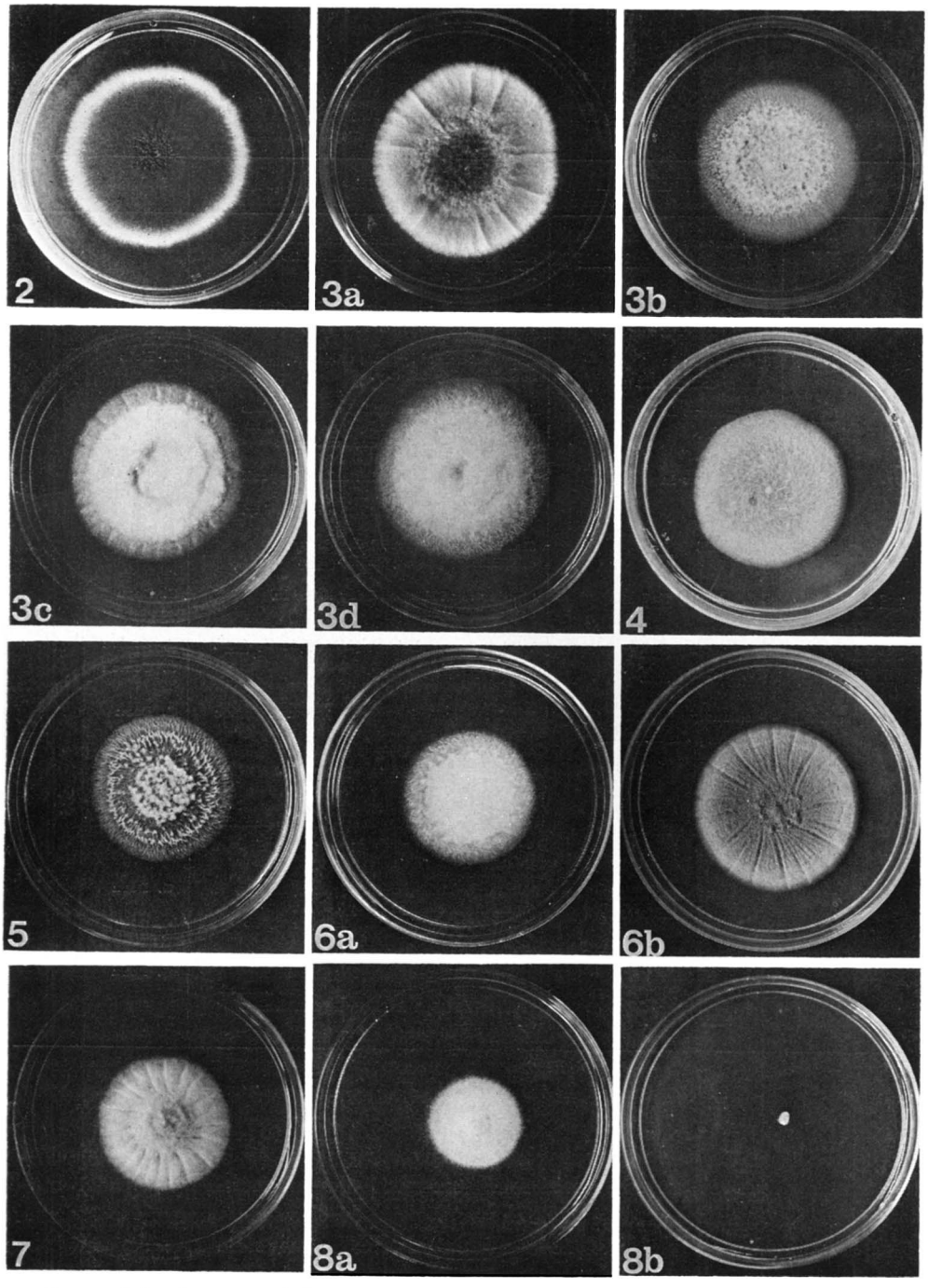

FIGURE 2–8.—Gross morphology of fluffy colonies grcwn on complete medium in 15 cm Petri dishes. The colonies shown in figures 6a and 8a were grown for 6 days at 25°C, while all of the other colonies were grown for 4 days at 37°C. Figure 2: *bi1*. Figure 3a, 3b, 3c and 3d: *flu1*, *flu3*, *flu4* and *flu13*. Figure 4: *flu2*. Figure 5: *flu7*. Figure 6a and 6b: *flu12*–25°C and *flu12*–37°C. Figure 7: *flu11*. Figure 8a and 8b: *flu8*–25°C and *flu8*–37°C.

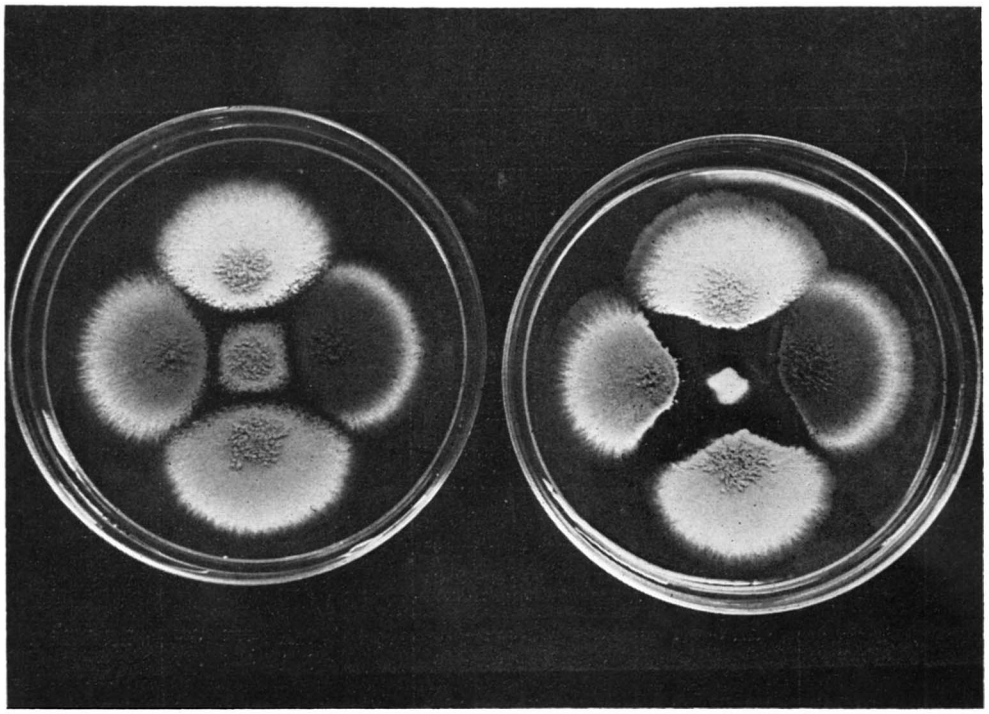

FIGURE 9.—Inhibitory effect of *flu8* on four non-fluffy strains. Left plate: Center, *y*; *nic2 facA303 ribo5*. Right plate: Center, *y*; *nic2 facA303 ribo5*; *flu8*. Both plates: Top, MSE; left, *y*; *nic2 facA303 ribo5*; right *bi1*; bottom, *ad23*; *facA3*; *cha*.

MSE *su1flu7; flu7* now carries an alkaline- acid phosphataseless mutation; MSE *su2flu7; flu7* carries an acid phosphataseless mutation; and MSE *flu7; rv5* no longer expresses the *facA303* mutant present in MSE *flu7*. The symbol *rv5* indicates that this strain is a revertant of MSE *flu7* but that the genetic basis of the reversion event is unknown.

Effect of various chemicals: The fluffy strains were exposed to subtoxic doses of several different growth inhibitors for the following reasons: (1) to see if normal differentiation of the conidial apparatus could be at least partially restored, (2) to see if the various fluffy strains could be separated into distinct categories on the basis of their response, and (3) to provide suggestive information as regards the causal biochemical lesion. Besides the drugs listed in Table 2, actidione, aminopterin, 5-fluorodeoxyuridine, iodacetate, and mitomycin C were tested and found to have no positive effect. From Table 2, however, it can be seen that one or more of the compounds known to affect protein synthesis restore almost normal conidiation to *flu2, 7, 12* and *13*; *flu11* responds solely to hydroxyurea, an antileukemia agent (STEARNS, LOSEE and BERNSTEIN 1963), while *flu1, 3, 4*, and *8* do not respond. In every case where a positive drug response was obtained, removal of the drug resulted in the complete restoration of the properties characteristic of the fluffy strain in question.

TABLE 2

Effect of various drugs on the differentiation of the fluffy variants

Fluffy variant*	Streptomycin (300 ug/ml)	Tetracycline (400 ug/ml)	Puromycin (400 ug/ml)	Choramphenicol (400 ug/ml)	Hydroxy urea (1000 ug/ml)
flu1	-	+	+	-	+
flu2	+++	++	+	++	±
flu3	-	-	+	-	-
flu4	-	-	-	-	-
flu7	++	++	-	-	-
flu8	-	-	-	-	-
flu11	-	-	+	-	++
flu12	++	±	-	-	-
flu13	-	-	++	-	±

+++ = Non-fluffy pattern and timing of differentiation; reference strain—*bi1*.
++ = Marked improvement in conidiation for poor and non-sporulating strains (*flu2*, *4*, *7*, *8*, *12* and *13*) or earlier formation of differentiated structures in delayed sporulating strains (*flu1*, *3*, and *11*).
+ = Slight improvement in level of conidiation or time of appearance of differentiated structures.
— = Fluffy–no improvement in the extent of conidiation or time of appearance of differentiated structures.
* = Fluffy *8* and *12* were grown on complete medium at 25°C and scored after 8 days; all other strains were grown at 37°C and scored after 5 days. The drugs were dissolved into the medium and inoculated with hyphal tips.

Genetic analysis: Because many of the fluffy variants do not sporulate and several are cross-sterile, meiotic analysis has proved generally difficult and in several cases impossible. Furthermore, the invasive properties of these strains result in a swamping out effect of many wild-type strains. This latter characteristic creates technical difficulties both as regards meiotic and mitotic analysis. Heterocaryons frequently go completely fluffy with no conidiation, making it impossible to select for a diploid. In certain cases where a morphologically wild-type diploid has been isolated, it often became progressively fluffy on subculturing. In scoring nonsporulating fluffy colonies, one cannot resort to a replication technique and this in turn renders genetic analysis time-consuming and tedious.

Several genetically distinct stocks of *Aspergillus nidulans* have never yielded fluffy sublines. Heterocaryons and diploids of these particular strains in combination with a fluffy isolate usually had a wild-type phenotype. These properties indicated that there was a possible difference in genetic background of some of the stock strains and, furthermore, that the mutation responsible for the fluffy state was recessive to its non-fluffy allele.

In order to prepare the strains for haploidization studies and also to have a common reference for testing dominance or recessiveness, heterocaryons and diploids were constructed between the fluffy strains and the following non-fluffy strains: MSD, MSE and *bi1*. As indicated in Table 3, *flu1*, *3*, *7*, *11*, and *12* appear to be recessive while *flu3* is probably dominant in the non-fluffy strains. *flu2* and

TABLE 3

Dominance and recessiveness of the fluffy variants against three non-fluffy reference strains

Fluffy strain	Non-fluffy strain *biA* Heterocaryon	*biA* Diploid	MSD Heterocaryon	MSD Diploid	MSE Heterocaryon	MSE Diploid
flu1	+	+	+	+	+	+
flu2	+	+	±	±	0	0
flu3	0	0	+	+	+	+
flu4	0	0	±	±	—	*
flu7	+	+	0	0	0	0
flu11	+	+	+	+	+	+
flu12	+	+	0	0	0	0
flu13	—	*	—	*	—	*

+ = Non-fluffy morphology.
± = Intermediate phenotype: heterocaryons showed an abundance of aerial hyphae and reduced sporulation; diploids took on appearance of fluffy parent on subculturing.
— = Fluffy morphology.
0 = Not tested.
* = Diploid could not be isolated because heterocaryon failed to sporulate.

4 are unusual in that the heterocaryons show a considerable amount of aerial hyphae and little or no sporulation. Furthermore, the isolated diploids become progressively fluffy after repeated subculturing. *flu 8* could not be tested because this strain has no forcing markers.

A summary of the mitotic and meiotic analyses of the fluffy system is presented in Tables 4 and 5. The data suggest a minimum of five loci controlling the expression of fluffy (linkage group I, 1; linkage group II, 1; linkage group VIII, 3). The haploidization data (Table 4) support the premise that *flu11* suppresses the expression of *palA1* and that three of the MSE *flu7* revertants were caused by an extracistronic suppressor, located on either linkage group II or VIII. In the case of MSE *flu7*; *rv5* either the suppressor is located on linkage group I or else it represents a back mutation. The mitotic analysis of these revertants also confirms the finding that MSE *su1flu7*; *flu7* now carries a linkage group II *palc* mutation, that MSE *su2flu7*; *flu7* carries a linkage group II *pac* mutation and that MSE *flu7*; *rv5* either carries a linkage group I suppressor of *facA303* or that this locus has back mutated. The results of meiotic analysis provide tentative evidence for the location of the three fluffy genes on linkage group VIII and the one on linkage group I (Table 5). Meiotic analysis also confirms that the expression of the *palA1* mutation is suppressed amongst the fluffy progeny only. The meiotic location of the *flu7* suppressor mutations and the new *palc* and *pac* mutations is currently under investigation.

DISCUSSION

The most striking features of the fluffy variants are their abnormal differentiation and invasiveness. It is of some interest to note that there appears to be an inverse correlation between the extent of invasion and the level of differentiation, i.e., the less differentiated mutants invade more rapidly. For example, strains

TABLE 4

Mitotic analysis of the fluffy variants—haploidization

Diploid	Number of haploids analyzed*	Indicated linkage group for fluffy or suppressor locus	Remarks
(su1ad20) gal5 an1 lu bi1; flu1 / MSD	72 { 69 flu; 3 flu+ }	flu1: III, V, or VIII	Very few flu+ haploids; possible Translocation: III-V-VIII.
y; w2 thi4 ni3 ad3 bi1; flu2 / MSD	38 { 22 flu; 16 flu+ }	flu2: VIII	Difficult to haploidize.
ad23; facA3; cha; flu3 / MSD	79 { 43 flu; 36 flu+ }	flu3: VIII	Translocation: VI-VII (known to be present in non-fluffy parental strain.)
bi1; yg; flu4 / MSD	28 { 11 flu; 17 flu+ }	flu4: VIII	Difficult to obtain diploid and haploidize; heterocaryon and diploid tend to become complete fluffy.
MSE flu7 / paba1; fw facB101 ribo2 gal7	98 { 18 flu; 80 flu+ }	flu7: I	
MSE flu7 / bi1	58 { 17 flu; 41 flu+ }	flu7: I	
MSE su1flu7; flu7 / bi1	73 { 23 flu; 50 flu+ }; 40**	flu7: I su1flu7: II? (2 exceptions)	Showed segregation for a temperature sensitive linkage group II alkaline-acid phosphataseless mutant.
MSE su2flu7; flu7 / bi1	74 { 22 flu; 52 flu+ }; 35**	flu7: I su2flu7: II	Showed segregation for a linkage group II acid phosphataseless mutant.
MSE su4flu7; flu7 / bi1	56 { 10 flu; 46 flu+ }; 38**	flu7: I su4flu7: VIII	
MSE flu7 rv5 / bi1	66 flu+	-----	No segregation of fluffy and facA303.
y; palA1 gal1 phen2; flu11 / MSE	57 { 33 flu; 24 flu+ }	flu11: III or VIII	Possible translocation: III - VIII; flu+ haploids showed segregation for palA1; flu haploids did not.

* The proportion of *flu* : *flu*+ segregants does not necessarily reflect their relative frequency of occurrence. The cases of aberrant segregation are indicated in column 3.

** Only those segregants known to be carrying *flu7* could be used for determining the linkage group of the *su flu* loci. Since it was established that the gene for *flu7* was on linkage group I, location of an extracistronic suppressor on a different linkage group could be determined by observation of the segregation of the chromosomal markers among the *y bi1*+ haploids.

Fluffy *8*, *12*, and *13* could not be analyzed for technical reasons.

like *flu4* and *13* infiltrate rapidly into a non-fluffy colony and within a week will cover the entire surface of the invaded colony. The invasion is always accompanied with the formation of abundant aerial and undifferentiated hyphae. On the other hand, the more differentiated forms like *flu1*, *3*, and *11* move into an adja-

TABLE 5

Meiotic analysis of linkage group VIII fluffy variants—random spore

Cross	Total progeny analyzed	Fluffy allele ratio flu : flu+	Gene order and distance (± 1 s. d.)*
y; w2 thi4 ni3 ad3 bi1; flu2 pabaA; w3; arg3 facB101 ribo2	103	47 : 56	arg3 — 21±4 — ribo2 — 29±5 — flu2 arg3 — 37±5 — flu2
ad23; facA3; cha flu3 pabaA; w3; arg3 facB101 ribo2	186	90 : 96	arg3 — 19±3 — flu3 — 12±2 — ribo2 arg3 — 22±3 — ribo2
ad23; facA3; cha flu3 pabaA; fw facB101 ribo2 gal7	180	94 : 86	flu3 — 12±2 — ribo2
MSR flu7 riboA anA ad14 proA biA; w2; pyro4	198	94 : 104	anA — 11±2 — ad14 — 18±3 — flu7 — 33±3 — proA anA — 23±3 — flu7 — 44±4 — proA
y; palA gal phen2 flu11 ** pabaA; w3 arg3 facB101 ribo2	182	110 : 72	flu11 — 13±2 — arg3 — 19±3 — ribo2 flu11 — 28±3 — ribo2
y; palA1 galA phen2; flu11 ** MSD	70	40 : 30	flu11 — 25±5 — ribo2

* All progeny came from a single crossed cleistothecium; the allele ratios and percent recombination of the known markers did not differ significantly from the published values (DORN 1967). In those cases where it could be checked, the order based on the least frequent class (double crossovers) was consistent with the order based on recombination frequency. The standard deviations were determined by using the formula $\sqrt{npq}$.

** In both of these crosses, the *pal* mutation did not segregate amongst the fluffies, i.e., they were all *pal*+; the *flu*+ progeny showed normal segregation.

cent colony more slowly and there is an overall reduction in aerial hyphal formation.

Although evidence presented here suggests that the fluffy state is essentially under the control of nuclear genes, DORN, MARTIN and PURNELL (1967) have been able to pass the *flu2* properties into a non-fluffy strain via heterocaryosis. A recent cross involving *flu12* and a non-fluffy strain (*pabaA*; *fw facB101 ribo2 gal7*) has yielded 100% fluffy progeny whereas all the other genetic markers segregated. These results strongly suggest that cytoplasmic involvement must be considered for at least some of the fluffy strains and work along these lines is currently in progress.

The fluffy variants do indeed appear to represent a unique class of mutants in fungi. Their rapid growth and invasive properties separate them from the slow and often irregularly growing variants described by PITTENGER (1956), MCDOUGALL and PITTENGER (1966), GARNJOBST, WILSON and TATUM (1965), and GARNJOBST and TATUM (1967). Although some of the fluffies superficially resemble the mycelials of ROPER (1958), the mycelials, unlike the fluffies, were induced with acridine, are unstable, and unable to infiltrate into established wild-type Aspergillus colonies.

In the previous section it was suggested that *flu8* might actually be a contami-

nant fungus since it had lost all of its genetic markers and grows poorly at 37°C. However, unusual marker changes have been observed in *flu11* and the UV-induced revertants of *flu7*. Temperature-sensitive mutants are known to exist in *Aspergillus nidulans* (DORN 1967). Finally, *flu8* is morphologically similar to the other fluffy strains. Since *flu8* does not produce asexual or sexual spores, it is impossible to classify this organism by standard taxonomic procedures. An attempt is being made to prepare antibodies specific to Aspergillus and perhaps then, the question of origin can be resolved. In any case, the ability of *flu8* to produce what appears to be a diffusible anti-fungal compound warrants that it be described.

In an attempt to integrate the unusual genetic changes, the drug responses, the possible cytoplasmic involvement and the abnormal differentiation pattern of the fluffy variants, several hypotheses merit consideration. For example, several of the fluffy mutants (*flu2*, *7*, *12*, and *13*) show a positive response to chemicals known to operate at the level of translation and, hence, the pleiotrophic effects could be ascribed to generalized misreading of the genetic code. On the other hand, if cytoplasmic involvement could be substantiated, then the multiple changes could be the result of a type of "host conversion" which would be a secondary effect of the self-replicating particle, e.g., virus or mitochondrion. Finally, these pleiotrophic effects might be due to the alteration of differentiation *per se*. It is possible that the expression of enzyme activity may be determined not only by the structural and regulatory loci but also upon the level or direction in which differentiation proceeds. At this time, however, it would appear to be useless to speculate further since many of the basic assumptions have not been adequately verified. Furthermore, the data presented here suggest that there may be more than one mechanism responsible for the initiation of the fluffy state.

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SUMMARY

A total of nine morphological mutants, called fluffy, has been isolated in *Aspergillus nidulans*. These mutants display varying degrees of delayed differenitation and are capable of invading into established non-fluffy cultures. Four *flu* loci and two extracistronic suppressor loci have been tentatively identified. On the basis of morphological properties, drug response, and genetic differences, it has been possible to place each fluffy variant into a distinct category.

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