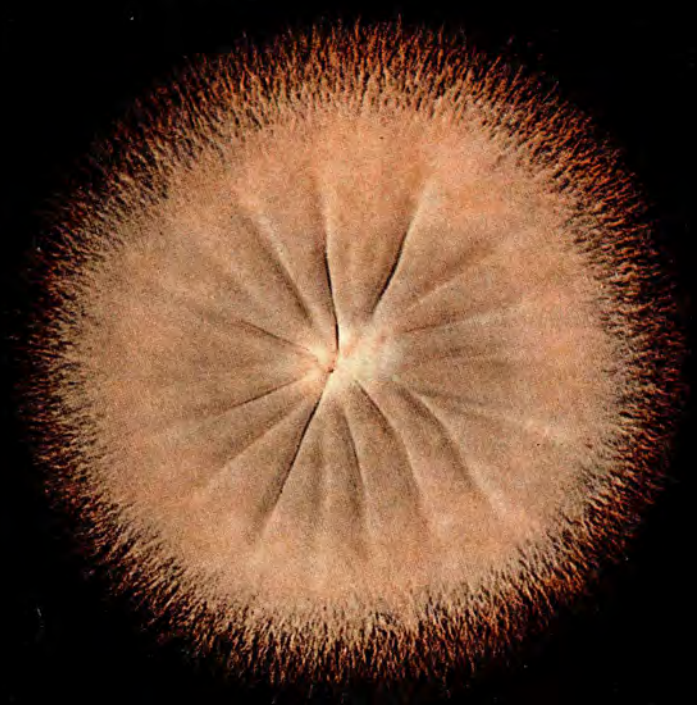
A 2547 E

mykosen



3/1970

1. März

Grosse Verlag GmbH. · 1 Berlin 31 (Halensee) · Kurfürstendamm 152

mykosen 13 (3) : 139—144 (1970) Eingegangen am 25. Juli 1969

Department of Biology, Medical Faculty
Olomouc, Czechoslovakia

Griseofulvin-resistant mutants in dermatophytes

2. Physiological and genetic studies

K. LENHART

Herrn Professor Dr. L. C h m e l zum 55. Geburtstag

In the first part of this paper the method of isolating mutants resistant to griseofulvin (GF) has been described in detail. The frequency of these spontaneous and UV-induced mutants in radom sets of spores from monosporic strain of *Microsporum gypseum* has been evaluated as well. In the second part presented some morphological, physiological and genetic characteristics of these mutants have been described.

Material, methods

1. Strains

Two compatible strains Z and 155 of the dermatophyte *Microsporum gypseum* (perfect stage *Nannizzia incurvata* STOCKDALE 1961) and 63 GF-resistant mutants isolated from strain 155 (12 spontaneous and 51 UV-induced mutants) were used. The characteristics of the wild strains and the isolation of the mutants were described in 1st part of this communication.

2. Evaluation of resistant mutants

Most of the characteristics were determined by the measurement of the growth rates on media of a different composition. The methods were adapted so as to enable a mutual comparison of the results. The dishes with standard inoculum were put into the thermostate (26° ± 2° C) and exactly after 10 days the diameters of the colonies grown were measured. Each value stated in the results represents the arithmetic mean obtained from the measurements of 3—5 colonies. The accuracy of the measurements to be estimated a 95 % confidence interval (C. I.) was calculated for each medium. The calculation of the accuracy of the measurement was based on the average values of the sample range (ECKSCHLAGER 1961, pg. 106).

In order to obtain a basic information concerning the growth rates Sabouraud glucose agar (SGA; formula according to HÜBSCHMANN and FRÁGNER 1962, pg. 21) was used; C. I. = ± 2.2 mm. The significance of the differences between the wild strain 155 and the mutants was determined by the variance analysis.

In order to determine marked physiological changes the minimal medium (MM) of the following composition was used: glucose 40 g; urea 2 g; Difco-agar 25 g; twice-distilled water 1 000 ml; C. I. = ± 5.1 mm. For a better comparability in each culture the quotient was calculated. i. e. the diameter of the colony grown on MM divided by the diameter of the colony grown on SGA (abbreviation MM/SGA).

The sensitivity to GF was evaluated by mycelial growth test (LENHART 1968 a). Each culture was inoculated on SGA (control) and on SGA with seven concentrations of GF (between 30—150 µg/ml for mutants). The value ED 50 was at first calculated by using the regression analysis, then it was graphically determined. C. I. for individual concentrations varies between 1.4—1.9 mm.

The morphology of the colonies was also evaluated on SGA after 10 days following the inoculation. In microscopical preparations obtained from the centre of the colony the production of spores was determined.

Resistant mutants of the strain 155 were crossed with the sensitive strain Z. The genetic analysis was made according to HEJTMÁNKOVÁ-UHROVÁ and HEJTMÁNEK (1965). These authors found that the complex of morphological differences between the strains 155 and Z is controlled by

a single gene. For this reason the colour of the colony was used as a visible marker (strain Z: brown colony, strain 155: cream colony). In the first experiment 50—80 colonies were randomly chosen from the smears of the only cleistothecium. Each of the colonies was inoculated three times on SGA (1) and on SGA + 50 µg GF/ml (2). On the medium (1) the morphology of each colony (155 or Z) was determined, on the medium (2) the sensitivity to GF (S — sensitive culture, R — resistant culture) was evaluated. In the second crossing 120—240 colonies were tested from each cleistothecium in the same way. The results obtained in this second crossing are stated in the results.

Results

In our set of 63 GF-resistant mutants only three morphological groups occurred. The difference between these groups is quite evident so that each mutant can be explicitly classified (**Table 1**). Six mutants only differed from the wild strain 155, i. e. the groups 2

Table 1: Macromorphology of GF-resistant mutants. In brackets: number of mutants significantly different from wild strain 155 in growth rate on SGA

Origin of mutants	Total	Macromorphology (groups)		
		1	2	3
Spontaneous	12	11	1	0
		(0)	(1)	—
UV-induced	51	46	4	1
		(4)	(4)	(1)

Group 1st: colony with no difference from wild strain 155.
Group 2nd: cream-coloured colony with markedly granular surface and reduced growth rate.
Group 3rd: whitish colony with felty surface and increased growth rate.

and 3. The production of spores has been preserved in all mutants. From the results it is to be seen that the changed morphology is always accompanied by a changed growth rate, too.

In **Table 2** the morphology, growth rate and the sensitivity to GF of some mutants were stated. Although the colony of the wild strain 155 grown on MM consists of a sparse mycelium, its diameter is approximately the same as of that grown on SGA (MM/SGA = 1). It is the mutant III/12, the only representative of the group 3 which significantly differs by the value MM/SGA from the wild strain 155. The development of resistance to GF is thus not accompanied by a qualitative change of requirements for growth substances.

All mutants are marked by the same resistance to GF: their ED 50 varies between 93—112 µg GF/ml; the differences between the individual values do not exceed the significance level. Comparing the wild strain 155 (ED 50 = 1.4 µg GF/ml) it can be seen that the resistance increased approximately hundred times. The diameters of the colonies of mutants grown in two concentrations of GF (45 and 70 µg/ml) are also stated in order to give a clearer idea of the course of the regression line which expresses the dependence between the concentration of GF and the growth inhibition. By both concentrations the same growth inhibition (about 70 %) was brought about.

Table 2: Morphology, growth rate and GF sensitivity of wild strain 155 and of several GF-resistant mutants.

D = average diameter of colony (mm). S = significant difference from growth rate of wild strain 155. Morphology — see table 1

Culture	Morphology	S G A	M M		45 µg GF/ml		70 µg GF/ml	ED 50 µg GF/ml
		D	D	MM/SGA	D	% (SGA = 100 %)	D	
W. s. 155	1	53,3	51,0	1,0	—	—	—	1,4
III/1	1	40,5 S	50,7	1,3	33,8	83,4	33,5	97
III/2	1	56,0	57,3	1,0	39,5	70,5	40,5	103
III/3	1	59,0	58,7	1,0	40,0	67,8	40,0	98
III/4	1	54,2	47,7	0,9	37,0	68,3	35,2	112
III/5	1	50,5	56,0	1,1	39,8	78,8	38,5	100
III/6	1	59,2	62,0	1,0	39,0	65,9	36,2	95
III/7	1	49,0	41,0	0,8	34,2	69,8	32,8	101
III/8	1	53,0	57,7	1,1	40,7	76,8	38,5	103
III/9	1	48,5	48,3	1,0	38,8	80,0	38,2	102
III/10	1	43,7	58,3	1,3	38,0	87,0	36,8	108
III/12	3	70,0 S	44,7	0,6	40,2	57,4	37,2	110
III/13	2	38,8 S	27,7	0,7	24,8	63,9	23,8	91
III/20	2	31,0 S	28,0	0,9	22,8	73,6	22,8	108
III/23	2	32,5 S	22,7	0,7	23,0	70,8	23,5	104

All resistant mutants were crossed with sensitive strain Z, however, in nine crossings only matured cleistothecia developed (**Table 3**). With a sufficiently high probability it can be assumed that in all of the nine mutants the resistance to GF is controlled by a single gene. This gene shows an independent assortment with the gene for morphology (155 or Z), the recombinants occurring with the same frequency as parental genotypes. Only in mutant X/3 a strong linkage between these two genes was found which was also proved in further crossings: the recombinants Z, R occur with a frequency of about 1 %. In the set of fertile mutants the resistance to GF is controlled by at least two genes, each of which may be in a different linkage group: one of them segregates independently with the gene for morphology and the other is linked with this gene. However, by both the mutant genes the same increase of resistance is caused.

Discussion

Our results represent the first step in the attempts to solve the problem of the resistance to GF. They indicate that this problem is not only theoretical interest, but also of a great practical importance. The genetic aspect of the origin of resistance has not been studied in dermatophytes as yet. When interpreting our results we have taken regard to the knowledge obtained in other species of fungi. The working methods of the study

Table 3: Results of crossing between GF-resistant mutants and sensitive wild strain Z

Mutants in crossing	Morphology of mutants	N_i	Macromorphology and sensitivity of colonies isolated				χ^2 for	P
			Z, R	Z, S	155, R	155, S	1 : 1 : 1 : 1	
VIII/1	1	156	41	33	37	45	2,05	0,50—0,60
X/2	1	146	31	41	35	39	1,7	0,60—0,70
X/3	1	173	48	39	44	42	1,0	0,80
X/5	1	194	45	52	40	57	3,5	0,30—0,40
X/8	1	240	62	54	69	55	2,4	0,40—0,50
IX/1	1	176	43	38	49	46	1,5	0,60—0,70
XI/2	1	128	34	28	35	31	0,9	0,80—0,90
XI/3	1	154	0	73	81	0	(1 : 1) (0,42)	0,50—0,60
XI/6	1	120	25	29	35	31	1,7	0,60—0,70

N_i = total number of colonies isolated and tested. R = resistant; S = sensitive, Z, 155 = macromorphology of colonies isolated according to the visible markers (see text)

of genetic control of resistance can be as a rule divided into the following three experimental stages:

The first stage begins with obtaining resistant mutants which are then crossed with sensitive strains. In each mutant the number of factors controlling the resistance are determined. Such are the papers dealing with the development of resistance of yeasts to copper (BRENES-POMALES et al. 1955), to cadmium (MIDDLEKAUFF et al. 1956), to actidione (MIDDLEKAUFF et al. 1957), to caffeine and nicotine (LINDEGREN et al. 1959). In *Neurospora crassa* TEAS and HOROWITZ (1948) studied in this way the genetic determination of the resistance to canavanine, HSU (1962) to acriflavine, actidione and caffeine; HOWE and TERRY (1962) studied the resistance to acriflavine, actidione and to three organic fungicides as well. In *Aspergillus nidulans* WARR and ROPER (1965) analyzed the genetic control of resistance to iodacetate and teoquil. In most cases the authors have found that in individual mutants the development of resistance is due to one mutation only. In our experiments we have also found in the group of fertile mutants that the resistance to GF is controlled by a single gene.

The work in the second stage is focused on the problem how to determine in the genotype of the organism under study all possible factors influencing the resistance to a certain drug. A considerable number of independently isolated mutants is mutually crossed, the individual factors are identified and their linkage relationships are determined. The hitherto information has shown that in microorganisms the resistance to drugs is mostly controlled by several genes. As a rule, several alleles can also be determined showing different levels of resistance and different manifestations in diploid or heterokaryotic state (dominance, recessivity). The effect of the individual factors can be mutually increased so that a different resistance in different genotype combinations is caused. In bacteria CAVALLI and MACCACARO (1952) have proved the existence of such a multigenic system in the study of the resistance of *E. coli* to chloramphenicol. The

Table 4: Genetic control of resistance to various drugs — summary of published data

Species	Resistance to	Number of factors	References
Hypomyces solani f. cucurbitae	chlorinated nitrobenzene	5 (probably more)	Georgopoulos (1963) Georgopoulos and Panopoulos (1966)
Aspergillus nidulans	acriflavine	2 genes 3 alleles	Roper and Käfer (1957)
	fluoroacetate	8	Apirion (1965)
	fluorophenyl-alanine	4	McCully — cit. ex Dorn (1967)
Saccharomyces cerevisiae	actidione	8 (and several modifiers)	Wilkie and Lee (1965) Cooper et al. (1967)
	nystatin	3 (and several modifiers)	Ahmed and Woods (1967)
	ethionine	3	Cherest et al. (1966) Robinchon-Szulmajster et al. (1966)
	5-fluorouracil	4	Lacrouette and Slominski (1964)
	copper	1 gene 3 alleles	Antoine (1965 a, b)
	copper	1 gene 2 alleles	Seno (1962)
Saccharomyces — various strains	lithium	5	Laskowski (1956)
	cadmium	1 gene 2 alleles	Nakamura (1964)

data concerning fungi are summed up in **Table 4.** In genetic analysis of GF-resistant mutants we have tentatively identified 2 loci in different linkage groups. Neither of nine fertile mutants shows any morphological differences from the wild strain 155. We have as yet not succeeded to explain the genetic nature of the change in morphology in the groups 2 and 3. Group 2 occurs both in spontaneous and in UV-induced mutants. It will be necessary to determine whether in this case another, i. e. a third gene for the resistance of GF is present which at the same time manifests itself morphologically.

In the third experimental stage the mechanisms of resistance on biochemical level is analyzed. In this respect no complete data for any drug have been obtained as yet. Most findings have been obtained in the study of resistance to antimetabolites, such as to ethionine, 5-fluorouracil and p-fluorphenylalanine. The minimum prerequisite for the construction of a working hypothesis is the knowledge of the primary place of effect of the drug in question. In such a way e. g. Ahmed and Woods (1967) proceed in the case of the antibiotic nystatin. However, the findings concerning GF in this respect are quite insufficient (Lampen et al. 1966, Huber 1968).

Summary

In this paper the results of the study of 63 GF-resistant mutants derived from sensitive wild strain 155 of *Microsporum gypseum* are summed up.

1. 6 mutants only differed from the wild strain by the character of the colony. The production of spores was preserved in all mutants.
2. All resistant mutants grew on minimal medium in the same way as the wild strain. The origin of resistance to GF is not accompanied by a qualitative change in the requirements for growth substances.
3. The increased resistance to GF is the same in all mutants: their values ED 50 vary between 93—112 µg GF/ml.
4. In the set of independently isolated mutants the resistance to GF is controlled by at least two genes, probably in different linkage groups. By both mutant genes the same resistance level is caused.

References

1. AHMED, K. A. and WOODS, R. A. (1967): Genet. Res. 9 : 179—193.
2. ANTOINE, A. (1965 a): Exp. Cell Res. **37** : 278—291.
3. ANTOINE, A. (1965 b): Exp. Cell Res. 40 : 570—584.
4. APIRION, D. (1965): Genet. Res. 6 : 317—329.
5. BRENES-POMALES, A., LINDEGREN, G. and LINDEGREN, C. C. (1955): Nature **176** : 841—842.
6. CAVALLI, L. L. and MACCACARO, G. A. (1952): Heredity 6 : 311—331.
7. COOPER, D., BANTHORPE, D. U. and WILKIE, D. (1967): J. Mol. Biol. **26** : 347—350.
8. DORN, G. L. (1967): Genetics **56** : 619—631.
9. ECKSCHLAGER, K. (1961): Chyby chemických rozborů. Praha.
10. GEORGOPOULOS, S. G. (1963): Phytopathology **53** : 1086—1093.
10. GEORGOPOULOS, S. G. and PANOPOULOS, N. J. (1966): Canad. J. Genet. Cytol. **8** : 347 349.
12. HEJTMÁNKOVÁ-UHROVÁ, N. and HEJTMÁNEK, M. (1965): Mycopathol. Mycol. appl. **25** : 183—194.
13. HOWE, H. B. and TERRY, C. E. (1962): Canad. J. Genet. Cytol. 4 : 447—452.
14. HSU, K. S. (1962): Rec. Gen. Soc. Amer. **31** : 92.
15. HUBER, F. M. (1968): Griseofulvin. In: Antibiotics, vol. I, 181—189. Springer-Verlag.
16. HÜBSCHMANN, K. and FRÁGNER, P. (1962): Dermatofyta a kožní choroby jimi vyvolané. Praha.
17. CHEREST, H. and ROBINCHON-SZULMAJSTER, H. (1966): Genetics **54** : 981—991.
18. LACROUTE, F. and SLONIMSKI, P. P. (1964): C. R. Acad. Sc. Paris **258** : 2172—2174.
19. LAMPEN, J. O., MCLELLAN, W. L. and EL-NAKEEB, M. A. (1966): Antimicrob. agents Chemoterap. 1006—1015.
20. LASKOWSKI, W. (1956): Genetics **41** : 98—106.
21. LENHART, K. (1968 a): Mykosen **11** : 195—204.
22. LENHART, K. (1968 b): Resistence dermatofytů ke griseofulvinu. Olomouc. Thesis.
23. LENHART, K. (1968 c): Mycopathol. Mycol. appl. **36** : 150—160.
24. LINDEGREN, C. C., LINDEGREN, G. and DESBOROUGH, S. (1959): J. Gen. Microbiol. **20** : 504—509.
25. MIDDLEKAUFF, J. E., HINO, S., YANG, S. P., LINDEGREN, G. and LINDEGREN, C. C. (1956): J. Bacteriol. **72** : 796—801.
26. MIDDLEKAUFF, J. E., HINO, S., YANG, S. P., LINDEGREN, G. and LINDEGREN, C. C. (1957): Genetics **42** : 66—71.
27. NAKAMURA, H. (1964): Japan. J. Genet. **38** : 261—269.
28. ROBINCHON-SZULMAJSTER, H. and CHEREST, H. (1966): Genetics **54** : 993—1006.
29. ROPER, J. A. and KÄFER, E. (1957): J. Gen. Microbiol. **16** : 660—667.
30. SENO, T. (1962): Japan. J. Genet. **37** : 207—217.
31. TEAS, H. J. and HOROWITZ, N. H. (1948): Genetics **33** : 127—128.
32. WARR, J. R. and ROPER, J. A. (1965): J. Gen. Microbiol. 40 : 273—281.
33. WILKIE, D. and LEE, B. K. (1965): Genet. Res. 6 : 130—138.

Address: Dr. K. LENHART, Department of Biology, Medical Faculty, Hněvotínská 3, Olomouc, Czechoslovakia