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Department of Biology and Department of Physiology, Medicine Faculty, Olomouc, Czechoslovakia

Studies on the Adaptation of Dermatophytes to Griseofulvin

K. Lenhart and E. Lenhartová

The role of each antibiotic for therapy is limited by the occurrence of resistant cultures of microorganisms. It is generally known that griseofulvin (GF) has played an important role in the therapy of dermatomycoses (Hübschmann and Frágner 1962). In order to estimate further perspectives of its application we therefore systematically study the question of the mechanisms and frequency of the GF-resistant cultures (Lenhart 1967, 1968 b). One of the way of the rise of resistant cultures in microbial population is phenomic adaptation (Bryson and Szybalski 1955). In filamentous fungi this phenomenon was studied by many authors (literature: Schnitzer and Grunberg 1957; Ashida 1965; Lenhart 1968 c). The fundamental knowledge can be summed up as follows:

1) To obtain a marked adaptation it is necessary to carry out repeated and long-termed transfers on the medium with a subletal or subinhibitional drug concentration.

2) An increased resistance is in fungi relatively smaller than in bacteria. This problem is discussed by Schnitzer and Grunberg (page 185).

3) The resistance of adapted cultures on the medium without drug is mostly in decrease. The deadaptation rate also depends on the applied strain of the fungus, the kind of drug and on the time of the adaptation training.

These facts are also in keeping with the data stated by the authors who studied the adaptation of dermatophytes to GF. Rosenthal and Wise (1960) obtained by repeated transfers on the medium with GF cultures with an increased resistance from two strains of *T. rubrum* and 1 strain of *M. canis*. Robinson et al. (1960) derived resistant cultures by the same manner. For these experiments they used *T. rubrum*, *T. mentagrophytes*, *M. audouini* and *M. canis*. Vidmar-Cvjetanovic et al. (1966, 1967) obtained a more resistant culture from *Achorion quinckeanum* by transfers on the medium with GF. Besides the test in vitro they also verified an increased resistance of the adapted culture by the experiment on the guinea pig. The failure of Roth et al. (1959) was very probably due to the application of an unsuitable method.

The above authors have unambiguously demonstrated the development of adaptability to GF in dermatophytes. We therefore did not consider it necessary to verify this conclusion in our strains. This paper is in direct connection with our previous communication (Lenhart 1968 b). Its aim is to contribute to a deeper knowledge of the dynamics of the adaptation process to GF.

Material and methods

Strains

Three strains of the dermatophyte *Microsporum gypseum* (Bodin) Guiart et Grigoraki (perfect state *Nannizzia incurvata* Stockdale) as well as the strain 139 of the dermatophyte *Microsporum cookei* Ajello (perfect state *Nannizzia cajetana* Ajello) were used. All strains were monosporically cleaned by the method after Hejtmánek and Lenhart (1964).

The procedure with GF, the preparation of the spore suspension and of Sabouraud glucose agar (SGA) as well as other fundamental procedures were stated in the previous communications (Lenhart 1968 a, b).

Block method

The procedure is based on the data given by PARRY and WOOD (1958, 1959 a, b) and on our own findings. Each strain was at first inoculated on Petri dish with a thick layer of SGA (6— 8mm). After a cultivation for fourteeen days blocks of medium with a mycelium of about 4 × 4 mm were cut out of the colony at the same distance from the centre. Each of these blocks was transferred into the centre of Petri dishes with three concentrations of GF in SGA (10, 30 and 60 µg/ml). In the same way the control was inoculated, too (SGA without GF). After fourteen days 2—4 Petri dishes were taken from each concentration, the grown aerial mycelium was shaken in distilled water and after filtration through the gauze the number of spores was modified. This spore suspension was used for testing the sensitivity by two following methods:

a) Mycelial growth test (LENHART 1968 a). The suspension was inoculated in Petri dishes with SGA and eight concentrations of GF (0,05—3,5 µg/ml). After a cultivation for ten days at a temperature of 26° C the diameter of the colonies was measured and by means of regression analysis the parametres of the growth straight lines and of the ED 50 value were calculated. In table summarizing the results values a and b are shown which are unbiased estimates of parametres α and β of linear regression function $y = \alpha + \beta x$; s_b is the mean error of the regression coefficient b. Standard deviation s_{xy} expresses the variability of empirically determined values round the regression line.

b) Sensitivity of spores (LENHART 1968 b). The diluted suspension was pipetted and spread on SGA (control) and on SGA + GF (2 and 4 µg/ml). On SGA the number of colonies was determined in 4 days, on the medium with antibiotics in 10 days. To determine the significance of the differences the arcsine transformation was used (URBACH 1964, page 131).

Biometry of spores

By the above block method the mycelium of the strain 139 on SGA with various concentrations of GF was obtained. From the mycelium fixed in 8 % formaldehyde microscopic preparations in Lugol solution were prepared. By measuring ocular (Meopta, ortho 15 x) and by the objective (magnification 43 x) the length, width and the number of macroconidial cells were determined. Methodical details were stated in the previous paper (LENHART and HEJTMÁNEK 1963 a).

The first experiment was carried out in order to obtain orientation results. The strain 139 was inoculated on SGA and further on SGA + 5 µg GF/ml. The mycelium was fixed in 13 days from inoculation. In the 2nd experiment five GF concentrations were used, i. e. 5, 10, 20, 30 and 40 µg/ml. The fixation was carried out in the 34th day since inoculation.

Table 1: The sensitivity of mycelium grown out on SGA with different concentrations of GF: results of mycelial growth test

Strain	GF (µg/ml)	a	b	s_{xy}	s_b	ED 50
155	Control	82,9	— 19,4	6,0	4,6	0,50
	10	91,3	— 23,6	6,2	4,8	0,57
	30	83,9	— 18,4	5,6	4,4	0,60
	60	86,6	— 21,3	7,4	5,8	0,52
Z	Control	97,2	— 20,8	4,2	3,3	1,62
	10	98,6	— 22,3	5,2	4,0	1,51
	30	94,9	— 20,3	5,9	4,6	1,66
	60	91,2	— 19,4	2,0	1,6	1,52
I-18	Control	67,9	— 17,5	3,4	3,4	0,11
	10	79,4	— 23,0	3,2	3,2	0,19
	30	78,4	— 19,3	3,9	3,9	0,30
	60	66,1	— 16,0	2,9	2,9	0,10

Results

After inoculating the medium with GF by small pieces of mycelium (about 1 mm^3) no growth could be determined in the concentrations from 20 µg GF/ml upward. By the application of block method the mycelium always grew on the same medium, as it could be gradually adapted. At first the sensitivity of the adapted mycelium to GF was evaluated. In Table 1 the parametres of the regression lines and the ED 50 values for the control and the individual experimental variants are stated. The differences between the individual ED 50 values are not significant, and therefore no increased resistance could be demonstrated by the mycelial growth test.

In Table 2 the results of the experiments are stated where the sensitivity of the individual spores was evaluated. The spores of the adapted mycelium formed colonies with a higher relative frequency than those of the control.

Our facts can be summed up as follows:

a) A significant increase of the spore resistance to GF can be determined after the only growth of the mycelium on the medium with this antibiotic.

b) The smallest concentration inducing this adaptability change amounts to 10 µg GF/ml.

c) The adaptive change is small, as it can be determined only by the evaluation of the sensitivity of individual spores.

GF is known by its morphological effects. We therefore tried to determine if the size and form of the macroconidia from mycelium grown on medium with GF were influenced (Table 3).These features are relatively constant and characteristic for each imperfect species of the dermatophytes. From the results it is evident that the differences between the control and experimental variants are very small. However, the variance analysis proved (Table 4, 5) that the presence of the antibiotic in the medium took a very considerable part in the variability of all three features. As expected this is valid especially for the first experiment.

For orientation purposes the relative frequency of such spores was evaluated the contoures of which differ from normal symmetrical spindle type. It was found that their frequency was on SGA with or without GF the same. In all cases only small shape deviations were concerned with.

Discussion

The resistance to antibiotics can develop by various mechanisms. In order to understand them completely it is necessary to pay attention not only to pronounced and permanent, but also to small and transient sensitivity changes. In order to define the resistance a modified definition after ASHIDA (1965) was used: the mycelium derived from the cells of the wild strain is designated as a resistant culture, if the following conditions are fulfilled:

a) the concentration of the agent must be increased to accomplish the same deleterious effect;

b) the same concentration of toxic agent causes a smaller effect than before.

The term "resistant culture" can be more precisely defined as a "culture more resistant than the wild strain". The principial methodical task was an exact evaluation of the fungus response to a definite antibiotic concentration. As GF is known by its fungistatic effect the inhibition of the mycelium growth and the formation of colonies by the spores ("sensitivity of spores") was taken as the sensitivity measure.

Table 2: The sensitivity of mycelium grown out on SGA with different concentrations of GF: results of evaluating spore sensitivity. C. I. = confidence interval (95 %). Sign. = significance of differences between control and experimental variants. S = significant; N = non-significant

		Relative frequency of spores which formed colonies					
		on SGA + 2 µg GF/ml			on SGA + 4 µg GF/ml		
Strain	GF (µg/ml)	%	C. I.	Sign.	%	C. I.	Sign.
Z	Control	43,2	28,7—58,0	—	2,1	0 — 8,4	—
	10	89,9	74,4—98,6	S	45,8	26,4—66,2	S
	30	57,2	42,0—72,2	N	44,9	30,0—60,4	S
	60	57,0	44,0—69,4	N	25,2	14,8—37,1	S
155	Control	0	—	—	0	—	—
	10	0,7	0 — 4,3	N	0,5	0 — 3,7	N
	30	63,8	48,5—77,8	S	1,3	0 — 7,1	N
	60	29,4	18,2—42,5	S	2,6	0,1— 8,4	N
I-18	Control	0	—	—	0	—	—
	10	65,2	44,5—83,3	S	1,4	0 —11,4	N
	30	71,8	53,0—87,2	S	31,8	15,5—50,9	S
	60	45,6	27,8—63,8	S	6,0	0,4—17,4	S

Table 3: The shape of macroconidia produced by adapted mycelium of M. c o o k e i, strain 139. Each value represents arithmetic mean in microns; n = number of measured macroconidia

Exp.	GF (µg/ml)	n	Length	Width	Number of cells
I	Control	150	49,18	16,40	7,7
	5	150	46,70	16,66	6,6
II	5	100	45,32	16,30	6,9
	10	100	51,58	17,07	5,6
	20	100	47,69	16,38	7,1
	30	100	47,63	15,39	7,1
	40	100	46,64	15,90	6,7

Table 4: Analysis of variance: the shape of macroconidia produced by adapted mycelium. 1st experiment

Feature	Source of variation	Sum of squares	Degrees of freedom	Variance	F	P
Length	Factor	781,83	1	781,83	5 250,85	< 0,01
	Remainder	44,37	298	0,15		
	Total	826,20	299			
Width	Factor	82,00	1	82,00	10 670,63	< 0,01
	Remainder	2,29	298	0,01		
	Total	84,29	299			
Number of cells	Factor	7 632,77	1	7 632,77	3 561,23	< 0,01
	Remainder	638,67	298	2,14		
	Total	8 271,44	299			

Table 5: Analysis of variance: the shape of macroconidia produced by adapted mycelium. 2nd experiment

Feature	Source of variation	Sum of squares	Degrees of freedom	Variance	F	P
Length	Factor	4,96	4	1,24	10,00	< 0,01
	Remainder	61,60	495	0,12		
	Total	66,56	499			
Width	Factor	0,36	4	0,09	17,34	< 0,01
	Remainder	2,57	495	0,01		
	Total	2,93	499			
Number of cells	Factor	164	4	41	5,34	< 0,01
	Remainder	3 801	495	7,68		
	Total	3 965	499			

The study of the adaptation processes to the toxic agents in filamentous fungi is from the methodical point of view very complicated. The authors determine as a rule which degree of resistance was obtained by transfers on the medium with griseofulvin. The cell free systems cannot be used as in the assay of enzyme activities. The troubles are due to a relatively complicated structure of the mycelium of filamentous fungi. It has not yet been determined whether the average number of the nuclei in the hyphae cells is changed by transfers on the medium with a toxic agent.

The ascospores, conidia and hyphae cells can be markedly differed by the number of the nuclei. It is not certain whether this fact is of any importance for the rise and transfer of an increased resistance. The role of extranuclear factors in adaptation has not been explained as yet (JINKS 1959, BRIAULT 1956).

In our last communication we determined (LENHART 1968 b) that the natural variability of spores enables the growth and vegetative production of the dermatophytes in a medium with a high concentration of GF. From a sufficiently dense spore suspension (about 10^7/ml) individual colonies on a medium with 20, 40, 80 and 160 µg GF/ml were produced, too. It is not certain whether the mycelial sensitivity of these colonies was increased in any way. The preliminary experiments suggested an increased resistance. In order to obtain a better comparison of the data the block method was used.

The mycelium that grew, owing to its natural variability (or by using the block method), on the medium with a high concentration of GF formed spores with a significantly increased resistance to GF. If these spores occurred in the environment with GF again, colonies with a greater frequency than the control were then produced.

The natural variability seemed to be a basis for adaptation process. The increased resistance was of course very small, as it could not be proved by the application of the mycelial growth test.

From our results it follows that the adaptation dynamics can be studied more precisely if the following condition is respected: the spores of the homokaryotic strain are considered as a statistical set where the drug sensitivity is random variable with the characteristic distribution of individual tolerances (LENHART 1968 b). The definition of the parametres of this distribution after individual transfers on the medium with toxical agents enables to describe the adaptation process quantitatively, i. e. to determine the gradual increase of the resistance depending on the defined environmental factors. The evaluation of the sensitivity can be simplified by the application of the germination test with drops on glass slides (PARRY and WOOD 1959). The definition of median effective dose ED 50 enables more accurate conclusions.

The adapted mycelium was characterized by means of morphological criteria. The strain 139 was chosen as the biometrics of its macroconidia had been studied in detail (LENHART and HEJTMÁNEK 1963 a, b; HEJTMÁNEK and LENHART 1964). It was determined that the presence of GF in the medium did not influence the morphogenetic processes of the macroconidial production.

Summary

Adaptation to GF was studied in three strains of *Microsporum gypseum* and in one strain of *Microsporum cookei*. After a single growth of the mycelium on the medium with GF a significant increase in the adaptability of spores was determined. This adaptability change is rather small. From the adapted mycelium the size of 800 macroconidia was evaluated. By GF the morphogenetic processes of the macroconidia development were not influenced.

Souhrn

Adaptace ke griseofulvinu byla studována u tří kmenů Microsporum gypseum a jednoho kmene Microsporum cookei. Již po jediném růstu mycelia na půdě s griseofulvinem bylo zjištěno signifikantní zvýšení odolnosti spór. Tato adaptivní změna je však velmi malá a přechodná. Z adaptovaného mycelia byla hodnocena velikost 800 makrokonidií. Bylo zjištěno, že griseofulvin neovlivnil morfogenetické procesy tvorby makrokonidií.

Zusammenfassung

Die Adaptation an Griseofulvin wurde bei 3 Stämmen von Mikrosporum gypseum und bei 1 Stamm von M. cookei untersucht. Nach einem einzelnen Mycelwachstum auf dem griseofulvinhaltigen Medium wurde eine signifikante Zunahme der Adaptabilität der Sporen festgestellt. Diese Veränderung der Adaptabilität ist ziemlich klein. Von dem adaptierten Mycel wurde die Größe von 800 Makrokonidien gemessen. Durch GF sind die morphogenetischen Prozesse der Makrokonidienbildung nicht beeinflußt worden.

Resumen

Se estudia la adaptación a G. F. de 3 cepas de M. gypseum y 1 cepa de M. Cookei. Luego de un único crecimiento del micelio sobre el medio con G. F. se pudo determinar un significativo aumento de la adaptabilidad de los esporos. Este cambio de adaptabilidad es bastante pequeño. Del micelio adaptado se evaluó el tamaño de 800 macroconidias. Los procesos morfogenéticos del desenvolvimiento de las macroconidias no fueron influenciados.

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Authors' address: Dr. Karel Lenhart, Department of Biology, Medicine Faculty Palacký University, Hněvotínská 3, Olomouc, Czechoslovakia

Dr. Eva [redacted]hartová, Department of Physiology, Medicine Faculty Palacký University,, Hněvotínská 3, Olomouc, Czechoslovakia