THE NATURE OF ANTITUBERCULOUS ACTION OF SULFISOXAZOLE. REPORT II

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In the first report of this study has it been indicated by us[1] that the generation time of *Mycobacterium tuberculosis* var. *hominis* is a function of the sulfisoxazole concentration. This result suggests that growth becomes visible even on media containing higher drug concentration, when a large size of inoculum is utilized, and most of the growth consists of no resistant variants but of sensitive cells. The purpose of the present paper is to know whether the suggestion is true or not.

Materials and Methods

The strain used in the study was *Mycobacterium tuberculosis* var. *hominis*, strain H37Rv. The medium and the methods of preparing cell suspensions were described in the first report of the study[1].

(1) Survivor curves

Each 0.02 ml of various dilutions of cell suspensions has been inoculated onto tubes containing various concentrations of sulfisoxazole, and the tubes inoculated have been incubated at 37°C. After 4 week incubation period, the numbers of colonies were calculated.

(2) Analysis of the population structure

Various cultures grown on various sulfisoxazole concentrations were kept until the end of the 6th week of incubation, which were used for making survivor curves. The cultures were shaken with glassbeads and suspended with saline. The suspensions were diluted with saline and 10^{0}–to 10^{-6}–dilutions were prepared. Each suspension was inoculated in 0.02 ml quantities onto each set of various drug concentrations. Each set consisted of 10 tubes.

After 4 week incubation, the numbers of colonies in control tubes were calculated. The numbers of colonies on media containing various drug concentrations (5, 10, 20 and 50 mcg per ml) were calculated after 6 week incubation, for the growth of the organism was delayed on these media. Ratios of the number of colonies on media containing the drug (=the number of survivors) against the number of colonies on media containing no drug (=the number of the total bacterial population) were determined to analyze the population structure, when the media were inoculated with the same cell suspension.

Results

(1) Survivors curves

The results are shown in Table 1. As shown in the table, tubes inoculated with 10^{-6}–dilution of the suspension gave discrete colonies, and the number of colonies was not affected by the drug concentration between 0 and 5 mcg of the drug per ml. Any statistically siginificant difference was not seen at 5 per cent level. A significant change was observed between 5 mcg and 10 mcg. However, it may be possible that the difference is not derived from selection of viable cells by the drug, for the size of colonies has decreased with increase of the drug concentration as will be shown in the following.

The size of colonies was not significantly different from each other on media containing 0, 0.5 and 1 mcg of sulfisoxazole per ml of medium. It decreased significantly on media containing 5 mcg of the drug and more significantly on media containing 10 mcg of the drug after 4 week incubation. At the time, any visible colonies were not observed on media containing above 20 mcg per ml. Indeed, growth was not visible on media containing 20 mcg of the drug, each of which had been inoculated with 10^{-3}–dilution of the suspension, *i. e.*, with ca. $112\times10^{3}=1.12\times10^{5}$ viable organisms. However, a thin membraneous growth was visible on media containing the same concentration of the drug, when each of the tubes had been inoculated with 10^{-2}–dilution, *i. e.*, 1.12×10^{6} viable organisms. On media containing 50 mcg of the drug growth was not visible, even when each of the tubes had been inoculated with 10^{-1}–dilution, *i. e.*, 1.12×10^{7} viable organisms. Growth was first observed on the media, when inoculation for each tube was made with the original suspension, *i. e.*, 1.12×10^{8} viable organisms. The growth on the media appeared not to be resistant population, for tubes inoculated with one-order less dilution must have given any growth, if the growth were derived from resistant variants. This suggestion has been proved to be correct by the analysis of the population structure.

Any visible growth was not observed in 10 tubes containing 100 mcg of the drug, even when the tubes had been inoculated with the original suspension, *i. e.*, 1.12×10^{8} viable organisms. Therefore, it has been considered that the bacterial population of the H37Rv strain does not contain any mutants to 100 mcg of the drug among 1.12×10^{9} viable organisms.

As has been mentioned above, the size of colonies

decreased with increase of the drug concentration, and the size of colonies increased with duration of incubation. After 6 week culture, growth became visible on media containing 20 mcg of the drug and inoculated with 10^{-6}-dilution. These findings are as has been considered from the results in the first report that the generation time is a function of the drug concentration.

Table 1. Numbers of survivors on various sulfisoxazole concentrations. (Test organism : *Mycobacterium tuberculosis* var. *hominis*, H 37 Rv. The number of colonies was determined after 4 week incubation period.)

Sulfisoxazole concentration (mcg/ml)	Dilution of inoculum				
	10^{-6}(#)	10^{-5}	10^{-4}	10^{-3}	10^{-2}
0	112.2±33.2(A)	M	M	M	M
0.5	97.7±22.1	M	M	M	M
1	110.5±10.6(B)	M	M	M	M
5	100.8±17.5(C)(*1)	M	M	M	M
10	82.1±16.6(D)(*2)	M	M	M	M
20	0.0	0.0	M	M	M(E)
50	0.0	0.0	0.0	0.0	M(F)
100	0.0	0.0	0.0	0.0	0.0

(#) (Mean of 10 replicates)±(Standard deviation).
M : Membraneous growth.
0.0: No visible growth on ten tubes.
(*1) Small colonies.
(*2) Minute colonies.
(A–F) These colonies were utilized for analyzing the population structure (See Table 2).

(2) Analysis of the population structure

Six week cultures grown on various drug concentrations, that is, 0, 1, 5, 10, 20, 50 mcg of sulfisoxazole, respectively, were analyzed for the population structure. The cultures were designated here as strains A, B, C, D, E, F, respectively (See Table 1). Strains A to D were obtained from the media inoculated with 10^{-6}-dilution and strains E and F were obtained from the media inoculated with the original cell suspension. The results are shown in Table 2. As shown in Table 2, the population structure was similar to each other in these 6 strains, and no significant difference was observed between these strains, although the strains had grown on various drug concentrations. Therefore, even growth occurring on higher drug concentrations and obtained first by inoculating concentrated cell suspension does not consist of resistant population but of sensitive population. The growth appears to have been derived from a few multiplication of many sensitive cells.

Discussion

The results obtained in the present paper indicate

Table 2. The population structure of the growth obtained in Table 1. (Test organism : *Mycobacterium tuberculosis* var. *hominis*, H 37 Rv. The numbers of colonies were determined after 6 week incubation period on media containing sulfisoxazole and after 4 week incubation period on media containing no drug. See Table 1 also.)

Strain (See Table 1)	Population structure			
	Sulfisoxazole concentration (mcg/ml)	Mean±SD (*1)	95% Confidence limits (*2)	95% Confidence limits (*3)
A	0	63.6±15.2	52.8～74.4	100%
	5	54.8±4.32	60.5～66.7	81.3～126
	10	50.2±6.71	58.8～68.4	79.1～129
	20	14.6±5.83	10.5～18.7	14.1～35.4
	50	0.0		
B	0	166.9±59.4	107.4～226.4	100%
	5	100.4±30.4	70.0～130.8	30.8～126
	10	104.3±35.6	79.0～129.6	34.8～121
	20	23.7±9.20	17.1～30.3	7.55～28.3
	50	0.0		
C	0	114.3±10.9	106.5～122.1	100%
	5	118.5±13.8	108.7～128.3	89.5～121
	10	82.6±13.1	73.3～91.9	60.0～86.8
	20	44.5±12.4	35.7～53.3	29.3～50.2
	50	0.0		
D	0	47.5±12.9	38.3～56.7	100%
	5	40.8±11.0	33.0～48.6	58.2～126
	10	22.3±15.1	11.6～33.0	20.4～86.0
	20	5.80±2.29	4.17～7.43	7.35～10.9
	50	0.0		
E	0	29.8±6.08	25.5～34.1	100%
	5	33.0±9.81	26.0～40.0	76.4～157
	10	11.1±3.69	8.50～13.7	24.0～53.8
	20	11.6±6.59	6.90～16.3	20.3～63.9
	50	0.0		
F	0	97.2±37.2	70.8～123.6	100%
	5	79.4±5.86	75.2～83.6	60.9～118
	10	47.3±17.9	34.5～60.1	28.0～84.6
	20	17.0±12.1	8.40～25.6	6.80～36.1
	50	0.0		

(*1) (Mean of survivors on ten replicates)±(Standard deviation).
(*2) 95% confidence limits of the mean of survivors derived from (Mean)±"t"×(Standard error).
(*3) 95% confidence limits of the ratio of survivors expressed as percentage of the total viable bacterial population : (B 1×100%/A 2) ···(B 2×100%/A 1).
A 1 and A 2 : Minimum and maximum of 95% confidence limits of the mean of survivors on media containing no drug.
B 1 and B 2 : Minimum and maximum of 95% confidence limits of the mean of survivors on media containing sulfisoxazole.

that a too small size of inoculum does not give any growth visible on higher drug concentrations, whereas a large size of inoculum does give a visible growth resulting from accumulation of growth of sensitive cells, and that a caution should be taken to consider that a visible growth may be produced from multiplication of sensitive cells, when the degree of sulfisoxazole resistance is determined fror clinical use.

Conclusion

The nature of tuberculostatic action of sulfisoxazole (3, 4-dimethyl-5-sulfanilamide-isoxazole) is in delaying the generation time, and the generation time is a function of the sulfisoxazole concentration. When a large size of inoculum is utilized, a visible growth can occur even on media containing higher drug concentrations. However, the growth does not consist of resistant population but of sensitive population, and the growth has been suggested to result from accumulation of delayed multiplication of sensitive cells. On the other side, when a small size of inoculum is utilized, growth remains not visible resulting from delayed multiplication.

When the degree of sulfisoxazole resistance of *M. tuberculosis* var. *hominis* is tested for clinical purpose, a caution should be taken to consider that growth occurring on higher concentrations of sulfisoxazole does not always mean the presence of sulfisoxazole resistance and it may consist of sensitive cells.

It appears the population structure of *M. tuberculosis* var. *hominis* is homogeneous with regard to sulfisoxazole sensitivity and it contains no mutants resistant to higher concentrations of sulfisoxazole.

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1) TSUKAMURA, M., NODA, Y. & YAMAMOTO, M.: The nature of antituberculous action of sulfisoxazole. Report I. Chemotherapy, 6 (3) : 165～167, 1958.