THE *IN VITRO* COMBINED EFFECT OF SULFISOXAZOLE WITH ANTITUBERCULOUS AGENTS. REPORT II

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(Received September 26, 1957)

The authors[1] studied previously on the *in vitro* combined effect of sulfisoxazole with antituberculous agents and reported that the effect consisted of the heterogeneous composition of bacterial population with regard to drug sensitivity and the retarding action of drugs on the generation time of the organism. This study concerned with the effect of sulfisoxazole with antituberculous agents inhibitory for a few individual cells of bacterial population. The present paper concerns with the combined effect of sulfisoxazole with antituberculous agents inhibitory for a greater part of individual cells of bacterial population and not inhibitory for resistant mutants and it concerns also with the mechanism of effect of sulfisoxazole in retarding the resistance to antituberculous agents.

Materials and Methods

Mycobacterium tuberculosis var. *hominis*, strain H 37 Rv, was used throughout the study. The medium used was the OGAWA's egg medium. Its composition was as follows : Basal solution (1% KH_2PO_4 and 1% sodium glutamate), 100 ml ; Egg, 200 ml ; Glycerol, 6 ml ; 2% aqueous solution of malachite green, 6 ml. The medium was poured in 8 ml quantities into tube of 18×170 mm and slanted by sterilization at 90°C for 60 minutes. Dihydrostreptomycin sulfate (Meiji Co.), isonicotinic acid hydrazide (Shionogi Co.), sodium *p*-aminosalicylate (Shionogi Co.), 4-acetylaminobenzaldehyde-thiosemicarbazone (=Tb 1/698, Takeda Co.), and 3, 4-dimethyl-5-sulfanilamide-isoxazole (Shionogi Co.) were used as streptomycin, isoniazid, PAS, tibione and sulfisoxazole, respectively. The drugs were added into medium prior to sterilization. After the sterilization, only the activity of streptomycin was regarded as one half of the activity added, for approximately one half quantities of streptomycin was adsorbed into protein by sterilization.

Cell suspension used as inocula were prepared as follows: 6 week-old-culture of discrete colonies was shaken with glass-beads for 10 minutes and suspended with saline. The suspension (the original suspension=10^0-dilution) was diluted with saline and 10^{-1} -to 10^{-7}-dilutions were prepared. The original suspension was inoculated in 0.02 ml quantities onto media containing streptomycin, isoniazid, PAS or tibione, respectively, with and without sulfisoxazole, and, on the other side, 10^{-4}-to 10^{-7}-dilutions were inoculated in the same quantities (0.02 ml) onto media containing sulfisoxazole alone and not containing any drug. The number of colonies was calculated after 6 weeks' incubation period.

Results

The results obtained are shown in Table 1. As shown in the table, number of streptomycin-and isoniazid-resistant colonies were not significantly changed by the presence or absence of sulfisoxazole. On the other hand, numbers of PAS-and tibione-resistant colonies increased significantly with increase of sulfisoxazole concentration, and it was observed, therefore, antagonism between PAS and sulfisoxazole as well as tibione and sulfisoxazole.

Discussion

The results obtained suggest that clinical combined use of PAS and sulfisoxazole or tibione and sulfisoxazole does not retard the emergence of PAS resistance or tibione resistance. The results appeared also to suggest, when observed from the standpoint of the number of survivors, that clinical combined use of streptomycin and sulfisoxazole or isoniazid and sulfisoxazole also might be not so much effective for retarding the emergence of streptomycin or isoniazid resistance. However, the effect of a drug on the emergence of an another given drug must be observed not only from the standpoint of the survivor number but also from the standpoint of the growth rate as indicated by us in the first report[1]. The colony size, *i.e.*, the colony diameter appears to be an index indicating the growth rate. The colony size of streptomycin-resistant colonies ranged from 1.5 to 2.0 mm on media containing 20 mcg of streptomycin alone as well as on media containing 20 mcg of streptomycin and 1 mcg of sulfisoxazole. However, it ranged from 1.0 to 1.8 mm on media containing 20 mcg of streptomycin and 5 mcg of sulfisoxazole in combination and from 0.8 to 1.0 mm on media containing 20 mcg of streptomycin and 10 mcg of sulfisoxazole in combination. Therefore, the total amount, *i.e.*, the total number of resistant mutants must be much smaller on media containing both streptomycin and sulfisoxazole than on media containing streptomycin alone. It would be expected that the combined use of streptomycin and sulfisoxazole in com-

Table 1. The *in vitro* combined effect of sulfisoxazole with streptomycin, isoniazid, PAS and tibione.

Size of inoculum	Medium (mcg/ml)	Average number of colonies per tube (*1)	No. of tubes	Significant change (*2)
0.02 ml of 10^{-6}-Dilution	No Drug	54.5 ± 10.2	10	
	SZ 1	42.1 ± 11.7	10	$P<0.025$
	SZ 5	35.5 ± 13.5	10	$P<0.005$
	SZ 10	23.8 ± 5.14	10	$P<0.001$
0.02 ml of 10^{0}-Dilution	SM 20	30.5 ± 8.01	20	
	SM 20-SZ 1	28.3 ± 7.06	20	$0.25<P<0.5$
	SM 20-SZ 5	31.9 ± 6.70	20	$0.25<P<0.5$
	SM 20-SZ 10	27.1 ± 5.43	20	$0.25<P<0.5$
0.02 ml of 10^{-1}-Dilution	IN 1	13.6 ± 7.48	20	
	IN 1-SZ 1	14.7 ± 8.33	20	$0.25<P<0.5$
	IN 1-SZ 5	15.7 ± 4.73	20	$0.25<P<0.5$
	IN 1-SZ 10	11.3 ± 5.65	20	$0.25<P<0.5$
0.02 ml of 10^{-1}-Dilution	P 10	6.70 ± 2.38	20	
	P 1-SZ 1	8.00 ± 3.67	20	$0.25<P<0.5$
	P 10-SZ 5	9.70 ± 2.60	20	$P<0.025$
	P 10-SZ 10	9.90 ± 3.32	20	$P<0.025$
0.02 ml of 10^{-1}-Dilution	TB 20	11.0 ± 4.75	20	
	TB 20-SZ 1	30.2 ± 4.76	20	$P<0.001$
	TB 20-SZ 5	(*3)	20	
	TB 20-SZ 10	(*4)	20	

(*1) (Mean) ± (Standard Deviation).
(*2) Significant change of mean number of colonies on media containing sulfisoxazole from mean number on media containing no sulfisoxazole.
(*3) Large colonies on membraneous growth.
(*4) Membraneous growth.
SM : Streptomycin; IN : Isoniazid; P : PAS; TB : Tibione; SZ : Sulfisoxazole.

bination retards the emergence of streptomycin resistance and the retarding effect results from the retarding effect of sulfisoxazole on the generation time of *Mycobacterium tuberculosis*[2]. The same phenomenon has been observed also with isoniazid.

The effect of snlfisoxazole on the *in vitro* emergence of streptomycin resistance and isoniazid resistance was first reported by the authors[3] in *Mycobacterium avium* and, thereafter, by NAITO[4], and OGAWA, *et al.*[5] in *Mycobacterium tuberculosis*. A mechanism of the effect appears to be interpreted by the reduction of absolute number of resistant mutants resulting from the retarding effect of sulfisoxazole on the growth of the organism.

Conclusions

The *in vitro* combined effect of sulfisoxazole with streptomycin, isoniazid, PAS and tibione (Tb 1/698) in concentrations selective for resistant mutants was observed on *Mycobacterium tuberculosis* var. *hominis*. The number of surviving streptomycin- and isoniazid-resistant colonies was not significantly affected by the presence of sulfisoxazole. The size of the resistant colonies, *i.e.*, the total amount of resisistant mutants was, however, reduced by the presence of sulfisoxazole. On the other hand, the number of surviving PAS- and tibione-resistant colonies was increased significantly by the presence of sulfisoxazole and an antagonism was observed between PAS and sulfisoxazole and between tibione and sulfisoxazole.

It was suggested that clinical combined use of streptomycin and sulfisoxazole or isoniazid and sulfisoxazole retards the emergence of streptomycin or isoniazid resistance, and, on the other side, clinical combined use of PAS and sulfisoxazole or tibione and sulfisoxazole does not retard the emergence of PAS or tibione resistance.

(The authors wish to express their appreciation to Dr. ROKURO KATSUNUMA, Director of the Obuso National Sanatorium, and Prof. SUSUMU HIBINO, Nagoya University, for their kind encouragement.)

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(See also report I (1).)