

FURTHER STUDIES ON THE MECHANISM OF THE COMBINED EFFECT OF ANTITUBERCULOUS DRUGS

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There are only a few papers concerning the mechanism of the combined effect of chemotherapeutic agents, although numerous reports have appeared concerned with the combined effect of different drugs on various bacteria. KLEIN and his co-workers⁽¹⁻³⁾ reported that the combined effect consists of the selective elimination of cells resistant to one drug by means of another drug. The authors⁽⁴⁾ reported in the previous paper that the combined effect has two factors. The one is selective mechanism on the number of viable bacterial population, and the other is the retarding effect on the growth rate of bacterial cells. According to the authors, the combined effect has appeared to be explained by doubling these two factors, without supposing any specific effect which might be newly produced by the combined use of drugs, excepting the case of antagonism. It is the purpose of the present paper to demonstrate the mode of action of combined drugs on the growth rate in media containing combined drugs.

Materials and Methods

Mycobacterium tuberculosis var. *hominis*, strain Aoyama-B, was used throughout the study.

OGAWA's egg medium was used throughout the study. The composition of the medium is as follows: Basal solution (1% sodium glutamate and 1% KH_2PO_4), 100 ml; Eggs, 200 ml; Glycerin, 6 ml; 2% aqueous solution of malachite green, 6 ml. The medium was poured in 8 ml amounts into tubes, 17 to 18×170 mm, and slanted by sterilization at 90°C for 60 minutes. Dihydrostreptomycin sulfate (Meiji Co.), isoniazid (Shionogi Co.), sodium *p*-aminosalicylate (Shionogi Co.), and 3,4-dimethyl-5-sulfanilamide-isoxazole (Shionogi Co.) were used as streptomycin, isoniazid, PAS and sulfoxazole, respectively. The drugs were dissolved in distilled water and added to media prior to the sterilization. The activity of streptomycin was calculated as one half of the added one after sterilization, while the activities of the other drugs were regarded as not altered by sterilization.

The generation time was measured according to YOUNG and YOUNG⁽⁵⁾. The organism was shaken with glass beads for 10 minutes and suspended in saline. Tenfold dilutions were prepared

from the suspension and each 0.02 ml of the dilutions were inoculated onto media by the use of a whirl loop delivering 0.02 ml⁽⁶⁾. Each set of media consisted of 50 tubes, and 5 different cell suspensions were used for inoculation. The time of the first appearance of visible growth was expressed as an average in 10 tubes. Incubation was made at 37°C. The generation time was estimated on media containing various single drugs as well as various combinations of two drugs.

Results

The results obtained are shown in tables. The prolongation of the generation time produced by the addition of single drugs or combined drugs is expressed as the difference of the generation time estimated on medium containing no drug and the generation time estimated on medium containing drugs added singly or in combination.

If one supposed that each drug acts independently in media containing two drugs, one would be able to make the following calculation:

The growth rate of the organism on medium containing no drug is designated as X , the growth rate on medium containing one drug, A, as X_a , the growth rate on medium containing another drug, B, as X_b , and the growth rate on medium containing both drugs, A and B, as X_c . If there were no specific action between two drugs, it would be expected that the ratio of the growth rate on medium containing two drugs against the growth rate on medium containing no drug (X_c/X) is a product of the ratios of the growth rate on medium containing single drugs against the growth rate on medium containing no drug ((X_a/X) and (X_b/X)); that is, the following correlation would exist.

$$(X_a/X) \times (X_b/X) = (X_c/X) \dots\dots\dots (1)$$

(Here, (X_a/X) , (X_b/X) and (X_c/X) correspond to "a, b, and c" in the previous report⁽⁴⁾.)

Now, if the generation time on medium containing no drug is designated as G , that on medium containing the drug A as $(G+a)$, that on medium containing the drug B as $(G+b)$, and that on medium containing both drugs as $(G+c)$, X_a , X_b , X_c and X are expressed as follows.

$$X = 1/G, \quad X_a = 1/(G+a), \quad X_b = 1/(G+b), \\ \text{and } X_c = 1/(G+c).$$

Incorporating these X_a , X_b , X_c and X into the equation (1),

$$(G/(G+a)) \times (G/(G+b)) = (G/(G+c)).$$

$$\therefore c = a + b + (ab/G) \dots \dots \dots (2)$$

The prolongation of the generation time produced by the addition of drugs is expressed as the difference between the generation time on medium containing drugs and the generation time on medium containing no drug. If there is no specific interaction between two drugs in combination, prolongation of the generation time estimated on medium containing any combination of two drugs would be similar to the prolongation calculated from the above equation. $c = a + b + (ab/G)$. The value G utilized in the calculation is an average of four estimates of the generation time.

Table 1 shows that streptomycin, PAS and sulfisoxazole prolong the generation time of the organism but isoniazid, in which the maximum concentration permitting the visible growth was used, does not prolong the generation time. Table 2 suggests that prolongation of the generation time estimated may be practically similar to that of the generation time calculated, if the error involved in estimating the generation time is considered. Nevertheless, there appear to be such tendencies that values estimated are larger than calculated ones in combination of streptomycin plus isoniazid and they are smaller than calculated ones in combinations of streptomycin plus sulfisoxazole and of isoniazid plus sulfisoxazole.

Table 1. Generation time of *M. tuberculosis* var. *hominis*, strain Aoyama-B, on OGAWA's egg medium containing various concentrations of single drugs. (The generation time was estimated according to YOUMANS and YOUMANS.)

Medium ($\mu\text{g/ml}$)	Growth Rate Constant	Generation Time (hours)
No Drug (Control)	0.716	10.1
Streptomycin 1 $\mu\text{g/ml}$	0.670	10.8 +0.7*
Streptomycin 2 $\mu\text{g/ml}$	0.523	13.8 +3.7*
No Drug (Control)	0.770	9.38
Isoniazid 0.01 $\mu\text{g/ml}$	0.800	9.02 -0.4*
No Drug (Control)	0.855	8.62
PAS 0.1 $\mu\text{g/ml}$	0.405	17.8 +9.2*
Sulfisoxazole 1 $\mu\text{g/ml}$	0.740	9.75 +1.1*
Sulfisoxazole 2 $\mu\text{g/ml}$	0.625	11.6 +3.0*
Sulfisoxazole 5 $\mu\text{g/ml}$	0.455	15.8 +7.2*

* Prolongation of the generation time produced by the addition of drugs. (Generation time on medium with drug)-(Generation time on medium without drug).

Table 2. Generation time of *M. tuberculosis* var. *hominis*, strain Aoyama-B, on OGAWA's egg medium containing various combinations of two drugs. (The generation time was estimated according to YOUMANS and YOUMANS.)

Medium ($\mu\text{g/ml}$)#	Generation Time (hours)	Prolongation of Generation Time (hours)	
		Estimated*	Calculated**
No Drug (Control)	9.61	0.0	0.0
INH 0.01-PAS 0.1	18.0	+8.4	+8.4
SM 1-INH 0.01	11.6	+2.0	+0.3
SM 2-INH 0.01	18.8	+9.2	+3.1
SM 1-PAS 0.1	24.0	+14.4	+10.6
SM 2-PAS 0.1	27.1	+17.5	+16.5
INH 0.01-SZ 1	9.75	+0.1	+0.6
INH 0.01-SZ 2	11.2	+1.6	+2.5
INH 0.01-SZ 5	15.4	+5.8	+6.5
SM 1-SZ 1	9.37	-0.2	+1.9
SM 1-SZ 2	13.1	+3.5	+3.9
SM 1-SZ 5	13.0	+3.4	+8.4
SM 2-SZ 1	11.0	+1.4	+5.2
SM 2-SZ 2	14.4	+4.8	+7.9
SM 2-SZ 5	16.6	+7.0	+13.7

INH: Isoniazid; PAS: Sodium p-aminosalicylate; SM: Dihydrostreptomycin; SZ: Sulfisoxazole (3, 4-dimethyl-5-sulfanilamide-isoxazole).

* Prolongation of the generation time actually produced by the combined use of two drugs. (Generation time on medium with a combination of two drugs)-(Generation time on medium without drug).

**Prolongation of the generation time calculated from the equation $c = a + b + (ab/G)$. ("a and b" are the prolongation of the generation time produced by single drugs, respectively. See text.)

Discussion

The results have shown that prolongation of the generation time estimated on medium containing two drugs is practically similar to that of the generation time calculated by supposing that there is no specific interaction between two drugs used in combination.

The results indicated that isoniazid has no significant retarding action on the growth rate of the organism. The fact suggests that any combination containing isoniazid will not produce a significant combined effect. On the other hand, the fact that streptomycin, PAS and sulfisoxazole have retarding effect on the growth rate suggests that combinations between these drugs may produce a significant elimination of visible growth on media containing such combinations, derived from accumulation of

prolongations of the generation time produced by each single drug.

The equation $c=a+b+(ab/G)$ also suggests that, if " a and b " are relatively small, the prolongation of the generation time produced by two drugs is similar to the addition of each prolongation produced by single drugs, and, if " a and b " are relatively large, it is much greater than the addition.

The authors will not exclude other factors of the combined effect as suggested by several authors^(7,8). Nevertheless, the authors believe that the combined effect is in greater part derived from two factors: the selective effect on viable bacterial numbers and the retarding effect on the growth rate of bacterial cells, and that each single drug acts practically independently even in combinations.

Summary

The mechanism of the combined effect of chemotherapeutic agents has been studied by utilizing antituberculous drugs as the test drugs and *M. tuberculosis* var. *hominis* as the test organism, and it has been indicated that prolongation of the generation time produced by the combined use of two drugs is practically similar to that of the generation time calculated by supposing that there is no specific interaction between two drugs used in combination. It has been thus suggested that each single drug acts independently in medium containing two drugs.

It has been also suggested that drugs retarding the growth rate, streptomycin, PAS and sulfisoxazole, may produce a significant elimination of visible growth in a combination of two drugs among them, and, on the other hand, that isoniazid having no significant action on the growth rate may not produce such significant elimination in any combination with other drugs.

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