SOME OBSERVATIONS ON THE MULTIPLE DRUG-RESISTANCE OF *MYCOBACTERIUM TUBERCULOSIS* OCCURRING IN SPUTA

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(Received August 29, 1958)

The problem of drug-resistance has become more and more serious in the course of chemotherapy of tuberculosis. Routine method of testing drug-resistance of *Mycobacterium tuberculosis* to antituberculous drugs normally consists of using media containing single drugs. When multiple drug-resistance had been shown on various media containing single drug by the method, a question would be raised whether the bacterial population tested consisted of a mixture of organisms resistant to each single drug or of organisms simultaneously resistant to multiple drugs.

Theoretically considering, the probability that each bacterium could be a mutant resistant to a drug in a given bacterial population should be of the same rate, *i.e.*, at random for each bacterium. Therefore, it may be considered that the probability for each bacterium to be a mutant simultaneously resistant to multiple drugs must be a product of probabilities for each bacterium to be a mutant resistant to single drug. Consequently, it is considered that ratio of mutants resistant to multiple drugs per total bacterial population is a product of ratios of mutants resistant to single drugs per total bacterial population. It is the purpose of the present paper to test whether the above theoretical consideration is true or not.

Materials and Methods

Sputa of a thirty years old female having far advanced pulmonary tuberculosis were used for the study. She had receiving successively a series of chemotherapy with streptomycin alone, PAS alone, streptomycin and PAS in combination, isoniazid alone, isoniazid and PAS in combination, and, at the last, streptomycin, PAS and isoniazid in combination in these ten years. She had been receiving triple combined chemotherapy with streptomycin (1g per day, 2g weekly), isoniazid (0.3g per day, 0.6g weekly) and PAS (8g daily), when the present study was made (from September 1957 to March 1958).

She appered to have not been improved by the therapy.

The study consisted of testing the composition of bacterial population with respect to various degrees of drug-resistance. By the test, administration of antituberculous drugs was ceased before twenty-four hours and during the gathering of sputa.

Sputa of the patient were gathered from the morning of the test day to the next morning and presented to the analysis of population structure of *Mycobacterium tuberculosis* in respect to drug-resistance. The sputum fluid gathered was added with the same amount of 5% KOH and thoroughly mixted with a pipette for 20 minutes. The sputum solution was then diluted with saline and 10^0 to 10^{-4} dilutions of the sputum solution were prepared. Each 0.02 ml of the dilutions was inoculated by a whirled loop onto media containing various concent-

Table 1. Population structure of *M. tuberculosis* occurring in sputum of a patient (November 29, 1957; Total amount of expectorated organisms per day : 3.73×10^6).

Medium	No. of survivors : Mean (95% confidence limits)*	Percentage of survivors	
		Estimated	Calculated**
		%	
No Drug	186.3(163.3-209.3)	100.0	
SM 10	151.4(121.7-181.1)	85.8	
SM 100	129.6(106.2-153.3)	73.4	
SM 1000	92.3 (80.0-104.6)	52.4	
IN 0.1	149.4(138.3-160.4)	84.8	
IN 1	49.4 (43.4-55.4)	28.0	
IN 10	18.3 (13.5-23.1)	10.4	
P 1	160.6(147.7-173.5)	91.0	
P 10	116.9 (89.8-144.0)	66.5	
P 100	0.0	<0.05	
SM 10-P 1	133.3(116.0-150.6)	75.8	78.1%
IN 0.1-P 1	142.8(122.7-162.9)	81.3	77.2
SM 10-IN 0.1	97.8 (75.4-120.2)	55.6	72.8
SM 10-IN 0.1-P1	132.8(117.2-148.6)	75.7	66.3
SM 10-P 10	89.0 (80.4-97.6)	50.5	57.0
SM 10-IN 1	40.6 (32.4-48.8)	23.0	24.1
IN 1-P 10	11.6 (7.6-15.6)	6.6	18.6
SM 10-IN 1-P 10	11.7 (6.5-17.2)	6.7	16.0

SM : Streptomycin ; IN : Isoniazid ; PAS.

(*) Mean of 10 replicates and 95% confidence limits derived from (Standard error)×(Student's"t").

(**) Derived from (Product of ratios of survivors on media with single drugs)×100%.

Table 2. Population structure of *M. tuberculosis* occurring in sputum of a patient (February 3, 1958 ; Total amount of expectorated organisms per day : 3.75×10^7.).

Medium	No. of survivors : Mean (95% confidence limits)*	Percentage of survivors	
		Estimated	Calculated**
		%	
No Drug	25.1(20.0-30.2)	100.0	
SM 10	24.6(21.2-28.0)	98.0	
SM 100	17.9(17.1-18.7)	71.5	
SM 1000	15.0(12.8-17.2)	59.9	
IN 0.1	20.3(17.9-22.7)	81.4	
IN 1	8.3 (7.8-8.8)	33.2	
IN 10	3.1 (2.6-3.6)	12.4	
P 1	22.6(19.0-26.2)	90.2	
P 10	10.2 (3.4-17.0)	40.8	
P 100	0.0	<0.4	
SM 10-P 1	20.9(14.7-27.1)	83.5	88.5%
IN 0.1-P 1	24.3(19.7-28.9)	97.5	73.2
SM 10-IN 0.1	17.5(14.8-20.2)	69.8	80.0
SM 10-IN 0.1-P1	18.9(13.8-24.0)	75.5	72.0
SM 10-P 10	4.1 (2.8-5.4)	16.4	40.0
SM 10-IN 1	5.2 (4.5-5.9)	20.7	32.5
IN 1-P 10	0.9	3.6	13.6
SM 10-IN 1-P 10	0.4	1.6	13.6

SM Streptomycin ; IN : Isoniazid ; P : PAS.
(*) Mean of 10 replicates and 95% confidence limits derived from (Standard error)×(Student's "t").
(**) Derived from (Product of ratios of survivors media with single drugs)×100%.

Table 3. Population structure of *M. tuberculosis* occurring in sputum of a patient (March 5, 1958 ; Total amount of expectorated organisms per day : 6.75×10^6.).

Medium	No. of survivors : Mean (95% confidence limits)*	Percentage of survivors	
		Estimated	Calculated
		%	
No Drug	50.4(41.7-58.3)	100.0	
SM 10	48.1(36.8-59.2)	95.5	
SM 100	38.5(31.2-46.8)	76.2	
SM 1000	38.9(29.5-48.5)	77.2	
IN 0.1	38.6(29.4-48.5)	76.3	
IN 1	0.6	1.2	
IN 10	0.3	0.6	
P 1	35.3(27.2-42.6)	69.9	
P 10	0.0	<0.2	
P 100	0.0	<0.2	
SM 10-P 1	26.3(21.8-30.1)	52.3	66.8%
IN 0.1-P 1	24.2(17.6-30.3)	48.1	53.3
SM 10-IN 0.1	30.2(23.1-36.9)	60.0	72.9
SM 10-IN 0.1-P1	21.6(16.8-27.1)	43.0	50.8
SM 10-P 10	1.7	3.4	<0.2
IN 1-P 10	0.0	<0.2	<0.2
SM 10-IN 1	0.5	1.0	1.1
SM 10-IN 1-P10	0.0	<0.2	<0.2

SM : Streptomycin ; IN : Isoniazid ; P : PAS.
(*) Mean of 10 replicates and 95% confidence limits derived from (Standard error)×(Student's "t").
(**) Derived from (Product of ratios of survivors on media with single drugs)×100%.

rations of single drugs as well as various combinations of two or three drugs, and tubes inoculated were stoppered with rubber-stopper and incubated at 37°C. Count was made after a six-weeks' incubation period and a series of the tubes inoculated with a dilution giving twenty to two hundreds discrete colonies were used as the basis of calculation[1].

1% OGAWA's medium was used for the test. Its composition is as follows : Basal solution (1% KH_2PO_4 and 1% sodium glutamate), 100 ml ; Eggs, 200 ml ; Glycerin, 6 ml ; 2% aqueous solution of malachite green, 6 ml. Medium was poured in 8 ml quantities into each tube, 170×17 to 18 mm, and slanted by sterilization at 90°C for 60 minutes. Dihydrostreptomycin sulfate (Meiji Co.), isonicotinic acid hydrazide (Shionogi Co.) and sodium para-aminosalicylate (Shionogi Co.) were used as streptomycin, isoniazid and PAS. The amount of streptomycin was regarded as one half of the original after the sterilization because of its adsorption to protein.

Results

Analysis of the population structure of *Mycobacterium tuberculosis* occuring in sputa of the patient was made three times (November 29, 1957 ; February 3, 1958 ; March 5, 1958). The results are small shown in tables 1 to 3.

As shown in the results, ratios of survivors of the total bacterial population estimated well agree with those calculated from the consideration described in the introduction, excepting only a few cases of ratios as not following the normal theory.

A sample of calculation is as follows: It is shown in table 1 that ratio of organisms resistant to 10 mcg of streptomycin is 0.858 and ratio of those resistant to 1 mcg of PAS is 0.910. Therefore, product of these ratios is $0.858\times0.910=0.781$. It is expected that ratio of organisms simultaneously resistant to 10 mcg of streptomycin and 1mcg of PAS

is 0.781. Ratio estimated of organisms simultaneously resistant to 10 mcg of streptomycin and 1 mcg of RAS is 0.758 as shown in table 1. The ratio estimated (0.758) is, therefore, similar to the ratio calculated (0.781).

The fact that ratios estimated well agree with ratios calculated, as shown in the tables, indicates that the consideration described in the introduction indeed occurs. Consequently, it may be considered that the ratio of organisms simultaneously resistant to multiple drugs is the product of ratios of organisms resistant to single drugs in a given bacterial population.

Discussion

Recently, ITO[2] and FUKUHARA[3] have claimed to test the method of drug-resistance test using media containing two or three drugs in combination. Further studies are requested to know whether the utilization of media containing multiple drugs is useful or not for clinical purpose. Nevertheless, the results obtained in the present paper indicates that the results of drug-resistance test using media containing multiple drugs can be prospected by carefully observing the results of drug-resistance test using media containing single drugs.

Summary

It has been indicated that the ratio of organisms resistant to multiple drugs is the product of ratios of organisms resistant to single drugs in a given population occurring in sputa of a tuberculous patient.

Aknowledgement

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 in the conduct of the present study.

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(References (1~3) are written in Japanese.)