THE QUANTITATIVE ANALYSIS OF THE DRUG RESISTANCE OF *MYCOBACTERIUM TUBERCULOSIS* IN PATIENTS. REPORT I

The Relationship between the Analysis of Isoniazid Resistance and the Chemotherapeutic Effect of Isoniazid

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The quantitative analysis on the composition of population of *Mycobacterium tuberculosis* with respect to isoniazid resistance was studied in sputa of patients by TOMPSETT[1], KOZAKAI[2], SATO[3-4], STEWART[5]. These studies concerned with the pattern of appearance of isoniazid-resistant organisms, and the studies dealing with the relationship between the quantitative analysis and the chemotherapeutic effect of the drug has not yet been presented. Owing to the complexity of the process of the emergence of isoniazid-resistant organisms under chemotherapy, careful quantitative observations utilizing a technique which permits enumeration of mutants of various degrees of resistance in direct cultures of sputum would be desirable for the judgement of the chemotherapeutic effect of the drug.

Materials and Methods

The percentage of acid-fast organisms in the sputum resistant to various concentrations of streptomycin, sodium-para-amino-salicylate (PAS) and isoniazid was determined in 16 patients with far advanced pulmonary tuberculosis with chronic cavities. Bi-monthly investigations were carried out during 6 to 16 months. During the period of observation the patients received the combined chemotherapy with isoniazid and PAS (isoniazid 300 mg twice or thrice weekly plus PAS 10 g or 3 g daily), and one of them (case 2) received chemotherapy with isoniazid 300 mg twice weekly, PAS 10 g daily and dihydrostreptomycin 1 g twice weekly. Four cases (cases 10, 12, 13, 14) of the patients received also chemotherapy with isoniazid alone (300 mg daily for 3 to 4 months) following cessation of the combined chemotherapy with isoniazid and PAS. The total amounts of isoniazid and PAS administrated during the observation period ranged from 20 g to 106 g and from 1,440 g to 4,100 g respectively, excepting one fetal case (isoniazid 4.5 g, PAS 700 g).

Method of the Quantitative Analysis of the Composition of Population with Respect to the Drug Resistance

Specimens of sputum were treated and hydrolyzed with an equal volume of 5% potassium hydroxide. After 15 minutes of pumping, the sputum hydrolyzed was diluted with saline and 10^0 (the original solution of sputum)-, 10^{-1}-, 10^{-2}-, 10^{-3}-, 10^{-4}-dilutions were prepared. Each 0.02 ml of each dilution were inoculated on the surface of OGAWA's KH_2PO_4 medium[6] (8 ml of slanted medium per tube) containing the following concentrations of dihydrostreptomycin, PAS, isoniazid in micrograms per ml of medium: 0, 1, 10, 100. These were then incubated and colony count was made at 6 weeks. From the counts calculation was made of the total bacterial population and of the proportion of cells in each culture resistant to each concentration of the drugs. Among colony counts obtained a count between 50 and 150 was taken as the basis of calculation, and if it were not possible, a count between 20 and 200 was taken. Owing to the tendency of *M. tuberculosis* to clumps, the colony counts represent viable units rather than individual cells.

Inoculation of tubes were made with a large whirled loop containing 0.02 ml. The method contains an error of adhering organisms to the loop. However, it has a beneficial point of obtaining well-dispersed colonies and of making possible to easily inoculate many tubes. On the other hand, inoculation with PASTEUR pipette does not contain the error above mentioned. However, it has a tendency of obtaining confluent colonies and of making calculation difficult. In the present study, the former method was preferred.

The composition of OGAWA's KH_2PO_4 medium is as follows: Basal solution (1% KH_2PO_4, 1% sodium glutamate), 100 ml; eggs, 200 ml; glycerol, 6 ml; 2% solution of malachite green, 6 ml. Each 8 ml of medium was poured into tube and slanted by sterilization at 90°C for 60 minutes. The activity of dihyrostreptomycin was calculated as one half of that added. Onto this medium one can inoculate the alkaline sputum solution without neutralizing.

Content of acid-fast organisms in sputum, blood sedimentation rate, temperature, weight and roentgenogram were observed and these clinical figures were compared at the beginning and the end of obser-

Case 3. S. K. ♀ 22 years.

Date	No. of organisms per ml of sputum	Percentage of resistant organisms per total viable organisms								
		SM (mcg)			PAS (mcg)			INAH (mcg)		
		1	10	100	1	10	100	1	10	100
7. 10. 1955.	6.5×10^7	100	100	23	52	52	0.01	0	0	0
12. 12. 1955.	4.6×10^5	100	100	70	52	9	0	0	0	0
12. 2. 1956.	3.8×10^5	100	100	100	100	0	0	0	0	0
9. 3. 1956.	7.6×10^6	100	78	36	74	77	0	0	0	0
23. 5. 1956.	3.0×10^6	100	87	60	74	0	0	0	0	0
12. 7. 1956.	3.6×10^6	100	*	5	100	0	0	0	0	0
12. 9. 1956.	1.6×10^6	100	100	10	8	2	0	0	0	0
14. 11. 1956.	1.7×10^6	100	100	77	100	0	0	0	0	0

* Contamination.

Aug. 1954–Feb. 1955, SM 52 g, PAS 1,800 g ; March 1955–Feb. 1956, INAH 50 g, PAS 2,900 g.

Content of acid-fast bacilli in sputum : no significant change.

Blood sedimentation rate : no change (73 mm～79 mm).

Temperature : slight reduction (febrile–subfebrile).

Weight : no change (38.0 kg～38.0 kg).

Roentgenogram : no change.

Results of chemotherapy : no change (fetal by hemoptysis, Jan. 7, 1957).

Case 5. T. H. ♀ 40 years.

Date	No. of organisms per ml of sputum	Percentage of resistant organisms per total viable organisms								
		SM (mcg)			PAS (mcg)			INAH (mcg)		
		1	10	100	1	10	100	1	10	100
9. 9. 1955.	1.0×10^7	100	100	0.07	100	2	0.1	100	25	0.01
21. 11. 1955.	3.7×10^6	100	50	0	56	0.5	0	100	30	0.15
15. 1. 1956.	1.0×10^6	100	50	0	100	88	24	100	67	0
23. 5. 1956.	1.1×10^7	100	100	0	33	36	22	17	3	0
12. 7. 1956.	1.1×10^6	69	48	50	58	58	70	62	5	0.03
12. 9. 1956.	3.5×10^5	100	100	0	100	100	100	100	100	0.6
14. 11. 1956.	1.2×10^6	61	61	0	68	57	13	78	35	0
17. 1. 1957.	4.3×10^6	70	65	0	100	100	100	100	23	0.02

July 1950–Sept. 1950, SM alone 40 g ; April 1951–Oct. 1951, PAS alone 500 g ; May 1952–Sept. 1952, PAS alone 500 g ; Dec. 1952–Feb. 1953, SM 50 g, PAS 1,800 g ; May 1953–Sept. 1953, INAH alone 12 g; Aug. 1954–Feb. 1955, SM 50 g, PAS 1,800 g ; Oct. 1955–March 1956, INAH 20 g, PAS 2,300 g ; May 1956–Feb. 1957, INAH 25 g, PAS 2,300 g.

Content of acid-fast becilli in sputum : no significant change.

Blood sedimentation rate : no significant change (63 mm～60 mm).

Temperature : no change (afebrile–afebrile).

Weight : slight reduction (33.0 kg～31.5 kg).

Roentgenogram : deterioration (In April 1956, a middle-sized cavity appeared in the left upper lobe. Cavities in the right lung not changed).

Results of chemotherapy : deterioration.

Remarks : Data shown in parentheses (A–B) indicate that the first (A) is the state at the beginning of observation and the second (B) that of the end.

Case 11. M. M. ♀ 43 years.

Date	No. of organisms per ml of sputum	Percentage of resistant organisms per total viable organisms								
		SM (mcg)			PAS (mcg)			INAH (mcg)		
		1	10	100	1	10	100	1	10	100
7. 10. 1955.	1.0×10^{7}	40	46	0.02	75	0.3	0	0	0	0
12. 12. 1955.	1.4×10^{6}	100	100	0.03	64	3	0.02	0.2	0.15	0.01
12. 2. 1956.	3.9×10^{5}	100	56	0.3	100	33	1.3	0	0	0
9. 3. 1956.	2.0×10^{5}	100	100	0	100	1	0.2	0.45	0.3	0.3
23. 5. 1956.	7.1×10^{5}	100	100	0.14	100	1.4	0.9	1.3	0.6	1.0
12. 7. 1956.	7.5×10^{5}	91	4	0	100	7	0.3	0.12	0.08	0.08
12. 9. 1956.	7.7×10^{4}	100	75	2	31	10	13	2.3	0.65	0.1
14. 11. 1956.	6.5×10^{5}	62	8	3	80	23	8	0.16	0	0
17. 1. 1957.	8.9×10^{5}	100	100	2.4	67	42	15	0	0	0

March 1952–July 1952, SM 40 g, PAS 1,800 g ; Aug. 1952–April 1953, INAH alone 18 g ; June 1953–Sept. 1953, INAH 9 g, PAS 3×130 g (intravenous administration) ; Jan. 1954–March 1954, INAH alone 12 g ; Oct. 1954–March 1955, SM 52 g, PAS 1,800 g ; Oct. 1955–Jan. 1957, INAH 50 g, PAS 3,840 g.
Content of acid-fast bacilli in sputum : no change.
Blood sedimentation rate : slight reduction (73 mm～55 mm).
Temperature : reduction (febrile–afebrile).
Weight : no significant change (42.0 kg～41.5 kg).
Roentgenogram : no change.
Results of chemotherapy : slight improvement.

Case 13. T. T. ♀ 28 years.

Date	No. of organisms per ml of sputum	Percentage of resistant organisms per total viable organisms								
		SM (mcg)			PAS (mcg)			INAH (mcg)		
		1	10	100	1	10	100	1	10	100
9. 9. 1955.	5.9×10^{5}	95	1	0.06	0.25	0	0	0.03	0	0
12. 12. 1955.	1.1×10^{4}	100	0	0	70	0	0	0	0	0
12. 2. 1956.	3.5×10^{5}	11	0	0	100	100	0	0	0	0
9. 3. 1956.	9.8×10^{5}	14	0	0	64	8	0	0	0	0
23. 5. 1956.	7.6×10^{5}	92	0	0	5	0.1	0	0	0	0
12. 7. 1956.	9.0×10^{4}	3	0.3	0	50	0.1	0	2.5	2	0.5
12. 9. 1956.	3.2×10^{6}	15	0	0	66	19	0.03	2	0.4	0.2
14. 11. 1956.	8.9×10^{5}	100	0.1	0	69	4	0	3	0.22	0.4
17. 1. 1957.	3.0×10^{6}	66	0	0	91	18	0	7	0.4	0

May 1951–Aug. 1951, SM alone 40 g ; Nov. 1951–April 1952, PAS alone 500 g ; Jan. 1953–June 1953, SM 10 g, PAS 500 g ; Nov. 1953–Jan. 1954, INAH alone 12 g ; Aug. 1955–March 1956, INAH 20 g, PAS 1,800 g ; April 1956–Sept. 1956, INAH 20 g, PAS 1,440 g ; Oct. 1956–Feb. 1957, INAH alone 50 g.
Content of acid-fast bacilli in sputum : no significant change.
Blood sedimentation rate : no significant change (36 mm～47 mm).
Temperature : no change (afebrile–afebrile).
Weight : no change (37.0 kg～37.0 kg).
Roentgenogram : no change.
Results of chemotherapy : no change.

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Results

Among the results obtained several cases are shown in tables.

During the observation period only 1 case (case 5) among these 16 patients contained above 10% of organisms resistant to 10 mcg of isoniazid per ml. Therefore, it has been considered that it is not practical to take the percentage of organisms resistant to 10 mcg of isoniazid as the basis of isoniazid resistance. Thus, the percentage of organisms resistant to 1 mcg of isoniazid (1 mcg-resistant cells) per total viable units has been taken as the basis of judgement of isoniazid resistance.

The course of appearance of 1 mcg-resistant cells could be divided into the following four groups.

A. During the observation period the percentage of 1 mcg-resistant cells was consistently 0%. 3 cases (cases 3, 4, 7.)

B. During the observation period the percentage of 1 mcg-resistant cells varied between 0 and 10% and in these cases a reduction of the percentage until 0% (remission of isoniazid resistance) was seen in all cases excepting only 1 case (case 8). 7 cases (cases 1, 8, 9, 10, 13, 14, 15.)

C. The percentage of 1 mcg-sesistant cells was 0% at the beginning of observation and it increased until 10% at the end of observation period. 2 cases (cases 12, 13.)

D. The percentage of 1 mcg-resistant cells was already above 10% at the beginning of the observation period and the percentage was maintained consistently. 4 cases (cases 2, 5, 6, 16.)

The therapeutic effect of the drug was judged by content of acid-fast organisms in sputum, blood sedimentation rate, temperature, weight, roentgenogram, and divided into the following 3 groups : improvement, no change, deterioration. All the patients had far advanced disease with chronic cavities. Accordingly, the effect was not so much large.

The relationship between the percentage of 1 mcg-resistant cells in bacterial population in sputum and the chemotherapeutic effect of isoniazid was as follows :

Group A : improvement 1 ;
no change 2 ; deterioration 0. (Total : 3)
Group B : improvement 3 ;
no change 3 ; deterioration 1. (Total : 7)
Group C : improvement 0 ;
no change 2 ; deterioration 0. (Total : 2)
Group D : improvement 1 ;
no change 0 ; deterioration 3. (Total . 4)

There is a tendency of improvement in groups A and B, and there is a tendency of deterioration in group D.

If one divides the patients into the following 2 groups : (I) the percentage of 1 mcg-resistant cells varied from 0 to 10% during the observation period (6~16 months); (II) the percentage of 1 mcg-resistant cells varied from 10 to 100% during the observation period, the relationship between the percentage and the chemotherapeutic effect of the drug is as follows :

Group (I) : impovement 4 ;
no change 7 ; deterioration 1. (Total : 12)
Group (II) : improvement 1 ;
no change 0 ; deterioration 3. (Total : 4)

Among the group (I) there is only 1 case of deterioration (case 8) in 12 patients and there are 4 cases of improvement (cases 1, 4, 11, 15.). On the other hand, among the group (II) there are 3 cases of deterioration (cases 2, 5, 6.) in 4 patients and there is only one case of improvement (case 16).

In the case of deterioration (case 8) in the group B or (I), the reduction of the percentage of 1 mcg-resistant cells until 0%, *i. e.*, the remission of the resistance did not occur and in this point the case was an exceptive one in the group. Therefore, if one divides the patients according to the existence of remission of resistance, the following relationship is obtainable.

a. No isoniazid resistance (the percentage of 1 mcg-resistant cells was 0% throughout the course of chemotherapy.) 3 cases (cases 3, 4, 7)

b. Resistance with remission (the percentage varied between 0 and 10% and the reduction of the percentage to 0% occurred during the course of chemotherapy. 5 cases (cases 1, 10, 11, 14, 15)

c. Emergence of resistance during the course of chemotherapy (=group C previously mentioned) 2 cases (cases 12, 13)

d. Resistance without remission (the percentage varied between 0.4 and 100% throughout the course of chemotherapy and the reduction of the percentage to 0% did not occur.) 6 cases (cases 2, 5, 6, 8, 9, 16)

Group a : improvement 1 (case 4) ; no change 2 (cases 3, 7) ; deterioration 0. (Total : 3)

Group b : improvement 3 (cases 1, 11, 15) ; no change 2 (cases 10, 14) ; deterioration 0. (Total: 15)

Group c : improvement 0 ; no change 2 (cases 12, 13) ; deterioration 0. (Total : 2)

Group d : improvement 1 (case 16) ; no change 1 (case 9); deterioration 4 (cases 2, 5, 6, 8). (Total: 6)

The results suggest that the existence of remission of resistance may be closely correlated with the effect of the drug on clinical symptoms.

Discussion

In view of the results obtained, it seems that there is a close correlation between the results of the percentage of 1 mcg-resistant cells in bacterial population of sputum and the chemotherapeutic effect of isoniazid. Therefore, the results emphasize the

need of the quantitative analysis of bacterial population for clinical use. Among 4 cases whose bacterial population showed consistently above 10% of 1 mcg-resistant cells, 3 cases had deterioration in spite of chemotherapy. However, there was one case of improvement in the patients. It appears to be drived of the complexity of isoniazid resistance. On the other hand, the caution for PAS resistance also should be made. This case had 19 to 67% of organisms resistant to 10 mcg of PAS in its bacterial population in the first course of chemotherapy, while the percentage decreased until 0% in the later course of chemotherapy. The effect of PAS may relate with the effect of chemotherapy.

When one divides the patients into two groups according to the percentage of organisms resistant to 10 mcg of PAS in the later course of chemotherapy, the correlation between the quantitative analysis of PAS resistance and the chemotherapeutic effect of the chemotherapy with isoniazid and PAS is as follows:

Group P-S: The percentage of organisms resistant to 10 mcg of PAS below 50%. Improvement 3; no change 4; deterioration 1.

Group P-R: The percentage of organisms resistant to 10 mcg of PAS above 50%. Improvement 2; no change 3; deterioration 3.

From these results, it would be considerable that the effect of chemotherapy with isoniazid and PAS depends rather on the existence of isoniazid resistance than that of PAS resistance. This problem will be discussed in detail in another report. The existence of remission of isoniazid resistance also appears to be closely correlated with the effect of the drug.

The percentage of organisms resistant to 1 mcg of isoniazid appears to be of low degree, because it is known that isoniazid concentration in the blood reaches several mcg per ml. The fact suggests that in pathological sites the selection of isoniazid-resistant organisms may be made by much lower concentrations of isoniazid than those expected. It seems of consideration that the existence of antagonists as pyruvic acid, hemin, *etc.* deals with the phenomenon.

CANETTI and SÄENZ[7] have shown that tubercle bacilli of varied degrees of streptomycin resistance may be isolated from different lesions in the same lung. Recent investigations by TURNBULL and STEWART[8] have shown that similar wide variation in isoniazid resistance can occur and supposed that sputum derived simultaneously from various sites may contain organisms of various degrees of resistance and different concentrations of the drug may depend on the sources of the sputum. Our results also show that wide variation in isoniazid resistance occurred in several cases (cases 1, 10, 11, 15). However, our results show that sputa contain organisms of much more uniform degrees of streptomycin resistance (a relatively homogeneous bacterial population with respect to streptomycin resistance) and those of much more different degrees of isoniazid resistance (a relatively heterogeneous bacterial population with respect to isoniazid resistance). The existence of homogeneous population with respect to streptomycin resistance (10 mcg per ml) has been shown in 13 among 14 patients excluding 2 patients who have received streptomycin therapy in the first course of the observation period. In these 13 cases, the percentage of organisms resistant to 10 mcg of streptomycin was practically invariably maintained. The fact suggests that bacterial population tested may be derived from the same site during investigations rather than that it may be derived from various sites. Previously, we reported that a streptomycin-resistant strain of *M. tuberculosis* var. *hominis* obtained by multiple steps of selection consists of very homogeneous population with respect to streptomycin resistance, and an isoniazid-resistant strain isolated by multiple steps of selection consists of considerably heterogeneous population with respect to isoniazid resistance, and a PAS-resistant strain isolated by multiple steps of selection consists of very heterogeneous population with respect to PAS resistance. Considering these results, it appears better to suppose that the heterogeneous composition of population with respect to isoniazid resistance occurring in the sputum is also derived from the nature of isoniazid-resistant *M. tuberculosis* itself.

Summary

The percentage of *Mycobacterium tuberculosis* in the sputum resistant to various concentrations of isoniazid, PAS and streptomycin was determined in 16 patients who had far advanced pulmonary tuberculosis with chronic cavities and received chemotherapy with isoniazid and PAS. The quantitative analysis of population of *M. tuberculosis* was carried out bimonthly during 6 to 16 months.

It has been indicated that there is a close correlation between the course of percentage of organisms resistant to 1 mcg of isoniazid per total viable cell units and the effect of the chemotherapy.

Among 4 patients whose bacterial population in sputum contained consistently above 10% of organisms resistant to 1 mcg of isoniazid, 3 patients showed deterioration during the chemotherapy. On the other hand, among 12 patients whose bacterial population in sputum contained below 10% of organisms resistant to 1 mcg of isoniazid throughout the course of the chemotherapy, there was only one case of deterioration.

Among 8 patients who showed no isoniazid resis-

tance or remissions of isoniazid resistance during the course of the chemotherapy, there was none of cases of deterioration. On the other hand, among 6 patients who had no remission of isoniazid resistance during the course of the chemotherapy, there were 4 cases of deterioration. Therefore, it has been also suggested that the occurrence of remission of isoniazid resistant, *i. e.* the reduction of the percentage of organisms resistant to 1 mcg of isoniazid to 0% may be closely correlated with the effect of the chemotherapy on clinical symptoms. The results emphasize the need of the quantitative analysis of bacterial population for clinical use.

It has been suggested that the heterogeneous composition of bacterial population in sputum with respect to isoniazid resistance may be derived from the nature of isoniazid-resistant *M. tuberculosis* itself.

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