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Studies on the Inhibitory Effect of Immunized Guinea Pig Sera on a Rapidly Growing *Mycobacterium*

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SUMMARY

To simplify examination of the tuberculostatic activity of immune sera by magnifying the difference between the growth of a test organism in normal and immune sera, attempts have been made to use *Mycobacterium*, Takeo strain, which grows very rapidly. Using a slide cell culture method a far more definite growth inhibition was exhibited by immune whole blood with Takeo strain than with Kamiike strain, a virulent *Mycobacterium tuberculosis* employed in the previous study. However, later it was found in slide and slide cell culture experiments that the growth of Takeo strain was not inhibited by immune plasma and serum specimens which definitely inhibited growth of Kamiike strain. It therefore appears that the inhibition of growth of Takeo strain by immune whole blood is not due to the action of the same humoral tuberculostatic factor as the factor active against Kamiike strain.

Thus it has been proved that Takeo strain cannot be used as the test organism for the detection or measurement of the tuberculostatic activity of sera of immunized or infected animals and humans.

INTRODUCTION

In previous papers (Kotani *et al.*, 1956; 1959) it was reported that sera taken from guinea pigs immunized with heat-killed tubercle bacilli exhibit a limited, but definite growth inhibiting activity against a virulent human type tubercle bacillus (a tuberculostatic activity) and that the active principle in immune sera is almost certainly an antibody and requires the cooperation of the complement for its activity. These studies were carried out by the slide culture method specifically modified to measure accurately the growth of tubercle bacillus in serum media. A serious technical difficulty of the method was that the tuberculostatic activity of immune sera persists for only one or two days on incubation as a result of inactivation of complement in the test serum medium and a difference of only 10 per cent between the growth of tubercle bacilli (log) in immune sera and that in normal sera must be considered as significant, in deciding on the presence or absence of tuberculostatic activity.

It was expected that use of a rapidly growing *Mycobacterium* as a test organism, which was sensitive to the tuberculostatic activity of sera from animals or humans immunized with tubercle bacillus, might overcome this technical difficulty. The results of experiments on this problem are presented here.

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MATERIALS AND METHODS

1. *Test organisms*

A rapidly growing *Mycobacterium,* Takeo strain, which was isolated at the Department of Tuberculosis, Research Institute for Microbial Diseases, Osaka University, was used as the test organism. This strain was formerly regarded as a species of *Mycobacterium avium,* but it has now been proved to be nonpathogenic to birds. A virulent human type tubercle bacillus, Kamiike strain, was used as a control. Both strains were serially subcultured on a modified Oka-Katakura medium* in this laboratory.

2. *Procedure for slide cell culture and slide culture*

Details of these procedures were described in the previous papers (Kotani *et al.,* 1953; 1956; 1959). Single cell suspensions of Takeo and Kamiike strains respectively were prepared from about 40 hour and 2 week old cultures on a modified Oka-Katakura medium, using a round bottomed flask containing glass beads. The bacterial suspensions thus prepared were repeatedly centrifuged at an appropriate speed to remove the clumps as completely as possible. The supernatant homogeneous suspension, which consisted almost exclusively to single cells, was used as an inoculum.

3. *Measurement of the growth of test organisms*

The method described in the previous papers was followed, but in the case of Takeo strain slight modifications were made: 1) many of the colonies become too large to count the number of constituent bacilli accurately after cultivation of 12 hours or longer and only approximate numbers could be estimated in such cases, and 2) because the colonies in the most peripheral parts of blood discs in slide cell cultures are liable to be disrupted during preparation of slides for microscopy, the growth in the peripheral parts was not considered in calculating the average number of test bacilli per microcolony per test blood disc.

4. *Immunization of guinea pigs*

Immunizing antigens were prepared from either 2 or 3 week old Kamiike cultures or from about 40 hour old Takeo cultures on a Oka-Katakura medium, by suspending them in autoclaved liquid paraffin at a concentration of 20 mg wet weight per ml and heating the suspensions at 70°C for 2 hours. Guinea pigs, weighing about 500 g, were injected intramuscularly with 0.5 ml each of this immunizing antigen preparation. The animals were used for experiments at different periods after immunization.

RESULTS

1. *Experiments with whole blood specimens*

Prior to experiments on serum specimens, a study was made by the slide cell culture method (which is simpler than the slide culture method) on whether whole blood specimens from guinea pigs immunized with tubercle bacilli inhibit growth of Takeo strain as well as Kamiike strain. Table 1 shows results of three representative experiments, in which the growth of Takeo strain in whole blood

* One of the most widely used media in Japan for isolation of tubercle bacilli from acid-treated sputum specimens of patients. The composition of the medium is as follows: KH_2PO_4 0.5 g, $Na_2HPO_4.12H_2O$ 0.5 g, sodium glutamate 1.0 g, glycerol 6 ml, 2 per cent malachite green solution 6 ml, whole egg homogenate 200 ml and distilled water 100 ml. Sterilized by heating at 85°, 80° and 80°C for 40 minutes on three consecutive days. Malachite green was omitted from the medium used in the present study.

specimens from 13 guinea pigs immunized (10 with Kamiike strain and 3 with Takeo strain) was compared with that in blood specimens from 12 control animals. The Table shows that the growth of Takeo strain is definitely inhibited by immune whole blood and that the degree of inhibition, that is, the difference between the growth in the immune and normal blood specimens is far greater than that observed in the previous studies in which Kamiike strain was used as test organisms.

Table 1. Inhibition of growth of a rapidly growing *Mycobacterium* (Takeo strain) by immune whole blood (slide cell culture)

Experiment No.	Growth of Takeo strain after cultivation for 24 hrs in				
	Normal whole blood		Immune whole blood		
	Guinea pig No.	Actual value (log)	Guinea pig No.	Days after immunization	Actual value (log)
1	597	2.14	573	20	1.88
	600	2.00	576	〃	1.60
	617	2.28	593	〃	1.74
	618	1.70	603	〃	1.40
	619	2.19	604	〃	1.78
2	634	2.28	576	30	1.40
	639*1	1.11	593	〃	1.45
	640	2.20	603	〃	1.65
3	543	2.09	556	40	1.40
	547	2.06	558	〃	1.54
	548	2.16	481*2	62	1.25
	549	1.99	482*2	〃	1.80
			491*2	〃	1.00

*1 An unusual case

*2 Guinea pigs immunized with heat-killed bacilli of Takeo strain not Kamiike strain (virulent *Myc. tuberculosis*)

It should be mentioned in this connection that in a few specimens of normal whole blood (for example, No. 639 guinea pig in Table 1), growth of Takeo strain was unusually low. This unusually low growth was observed in 7 out of total 57 non-immunized whole blood specimens examined in the present study. Phagocytosis may be responsible for this abnormality because 5 of the 7 unusual cases showed an unusually high rate of phagocytosis (cf. section 3).

Thus the expectations were partly realized, but the observed growth inhibition of Takeo strain in immune blood was not so complete as expected and a considerable growth was obtained even in blood specimens of immunized guinea pigs. To see why there is no complete inhibition of growth in immune blood, the growth of Takeo strain in both normal and immune whole blood was examined after various periods of cultivation. No appreciable inhibition of the growth by

immune whole blood was found during the initial 8 hours cultivation (Table 2). This delayed manifestation of the growth inhibition may explain why growth of

Table 2. Lag in manifestation of growth inhibition against Takeo strain by immune whole blood (slide cell culture)

Experiment 1

Blood specimens taken from	Guinea pig No.	Growth (actual value, log) of Takeo strain after cultivation for			
		4 hrs	8 hrs	12 hrs	20 hrs
Normal guinea pigs	510	0.34	0.82	1.26	1.76
	542	0.46	0.92	1.23	1.76
Immunized guinea pigs	464	0.42	0.88	1.00	0.95
	465	0.38	0.77	0.85	0.81
	470	0.42	0.90	1.10	1.16

Experiment 2

Blood specimens taken from	Guinea pig No.	Growth (actual value, log) of Takeo strain after cultivation for	
		24 hrs	72 hrs
Normal guinea pigs	542	1.97	2.02
	543	2.13	2.16
	547	2.15	2.12
Immunized guinea pigs	440	1.30	1.05
	472	1.19	1.00
	538	0.94	0.72

Takeo strain is not completely inhibited by immune whole blood specimens. An attempt to magnify the difference between the growth in normal and immune whole blood by continueing the cultivation for more than 24 hours also failed, for the growth of Takeo strain was maximal at 24 hours. This is probably due to the limiting amount of necessary nutrients in the blood specimens used in the slide cell culture.

Fig. 1 shows the growth of Takeo and Kamiike strains in whole blood taken from normal and immunized guinea pigs, from the results presented in Table 2 and from observations reported in the previous paper (Kotani *et al.*, 1953).

2. *Experiments with serum and blood plasma specimens*

The effect of immune serum specimens on growth of Takeo strain was also studied. Table 3 shows results of an experiment on the growth of Takeo and Kamiike strains in serum specimens from guinea pigs immunized with tubercle bacilli and in normal serum by the slide culture method. None of the 5 immune serum specimens, which partially inhibited growth of Kamiike strain, inhibited the growth of Takeo strain.

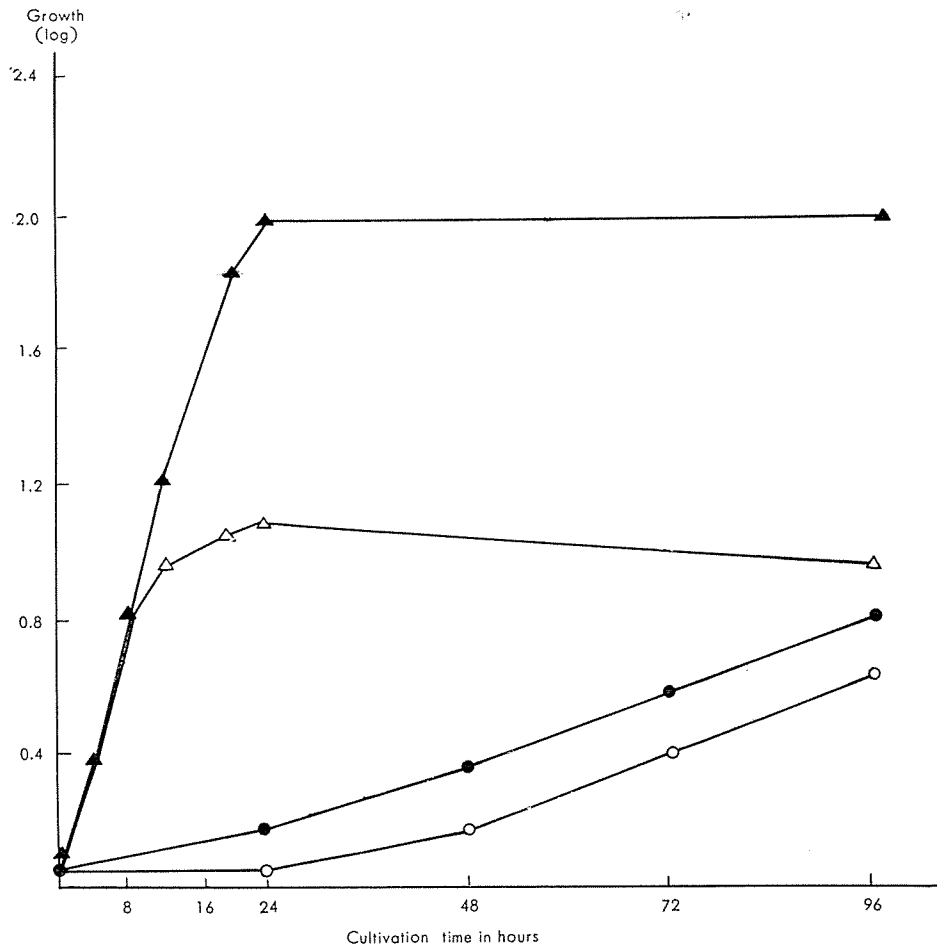

Fig. 1. Growth curves of Takeo and Kamiike strains in immune and normal whole blood

▲——▲ Takeo strain – normal whole blood
△——△ Takeo strain – immune whole blood
●——● Kamiike strain – normal whole blood
○——○ Kamiike strain – immune whole blood

Therefore the inhibition of growth of Takeo strain by immune whole blood may not be caused by the same humoral factor as the factor active against Kamiike strain. This idea is supported by the observation that there is no growth inhibition of Takeo strain by blood plasma specimens taken from immunized guinea pigs in which whole blood was definitely inhibitory (Table 4).

3. *Participation of phagocytosis in the inhibition of the growth of Takeo strain by immune whole blood*

The results described in the foregoing sections suggest that phagocytosis may

Table 3. Comparison of growth of Kamiike and Takeo strains in normal and immune serum (slide culture)

Serum specimens taken from	Serum No.	Growth (relative value*) after cultivation of	
		Takeo strain for 24 hrs	Kamiike strain for 3 days
Normal guinea pigs	1	96	99
	2	102	99
	3	103	98
	4	99	106
	5	99	99
Immunized guinea pigs	1	101	81
	2	95	82
	3	98	59
	4	91	73
	5	92	93

* Values calculated as a percentage of the average growth of either strain in 5 normal serum specimens

Table 4. Inhibition of growth of Takeo strain by immune blood plasma and whole blood (slide cell culture)

Whole blood or blood plasma specimens taken from	Guinea pig No.	Growth (actual value, log) of Takeo strain after cultivation for 20 hrs in	
		Blood plasma	Whole blood
Normal guinea pigs	648	1.60	1.96
	649	1.61	1.95
	650	1.57	1.93
Immunized guinea pigs	601	1.60	1.23
	618	1.70	1.61
	622	1.72	1.04

in some way be responsible for the inhibition of the growth of Takeo strain by immune whole blood. This was examined by arranging the protocols of some of the experiments presented in preceding Tables so that the growth of phagocytized and non-phagocytized bacilli could be calculated separately. The results summarized in Table 5 show that the growth of Takeo strain in immune whole blood specimens was significantly lower than that in control specimens, irrespective of whether the bacilli were phagocytized or not. No significant difference was found in the rate of phagocytosis (the number of colonies of phagocytized bacilli/total number of colonies) between immune and normal whole blood specimens. Therefore an increase in the proportion of phagocytized bacilli, whose growth is definitely lower than that of non-phagocytized bacilli, is apparently

not responsible for the inhibition of the growth of Takeo strain in immune whole blood specimens.

The result, that the growth of non-phagocytized bacilli was also definitely inhibited by immune whole blood, seems to conflict with observations on immune plasma and serum specimens. No satisfactory explanation based on experimental evidence has yet been found.

Table 5. Participation of phagocytosis in inhibition of growth of Takeo strain by immune whole blood (slide cell culture)

Exp. No.	Whole blood specimens taken from	Guinea pig No.	Rate of phago-cytosis	Growth (actual value, log) after cultivation for 24 hrs of Takeo strain		
				phagocytized	Non-phagocytized	Total
1	Normal guinea pigs	542	64	1.37	2.36	1.97
		543	33	1.10	2.31	2.13
		547	31	1.16	2.32	2.15
	Immunized guinea pigs	439	37	0.79	1.79	1.63
		440	25	0.75	1.41	1.30
		472	50	0.68	1.43	1.19
		538	55	0.73	1.20	0.94
2	Normal guinea pigs	543	47	1.21	2.34	2.09
		547	35	1.50	2.17	2.06
		548	26	1.66	2.25	2.16
		549	19	1.61	2.03	1.99
	Immunized guinea pigs	481*	38	0.69	1.37	1.25
		482*	60	1.52	2.04	1.80
		491*	28	0.67	1.08	1.00
		556	90	0.87	2.34	1.39
		558	67	0.94	1.97	1.55
3	Normal guinea pigs	648	72	1.65	2.31	1.96
		649	57	1.29	2.22	1.95
		650	51	1.30	2.15	1.93
	Immunized guinea pigs	601	68	1.03	1.48	1.23
		618	72	1.05	2.17	1.61
		622	60	0.73	1.31	1.04

* Guinea pigs immunized with heat-killed bacilli of Takeo strain not Kamiike strain.

DISCUSSION

An attempt has been made to simplify the measurement of growth inhibiting activity against tubercle bacilli of immune sera by use of a rapidly growing *Mycobacterium*, Takeo strain, as a test organism. The results by the slide cell culture method using whole blood specimens seemed promising. However, there was

no significant growth inhibition of Takeo strain by either plasma or serum specimens from immunized guinea pigs, although whole blood specimens from the same animals definitely inhibited growth. Therefore Takeo strain cannot be used as a test organism for the detection and measurement of a tuberculostatic activity of immunized sera of animals or humans.

The growth of Takeo strain is definitely inhibited by immune whole blood, however, and this is of interest and worth further investigation. It has been shown that this growth inhibition is not due to an increased rate of phagocytosis and that the growth of Takeo strain in immune whole blood, whether the cells were phagocytized or not, was significantly lower than that in normal blood. The finding that in immune whole blood the growth of non-phagocytized as well as phagocytized bacilli was inhibited as compared with growth in normal blood seems to be contradictory to the observations that no inhibition of growth occurred in either plasma or serum specimens. One possible explanation for these conflicting results is that a substance or substances which alone or together with other humoral factor(s) inhibit the growth of Takeo strain might be produced or released as a result of the interaction between inoculated bacilli and leucocytes in whole blood of immunized and sensitized guinea pigs. Further close investigation will be required to elucidate this point.

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