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Characterization of a Tuberculostatic Substance in Sera of Guinea Pigs Immunized with Heat-killed *Mycobacterium tuberculosis*[*1, *2]

SHOZO KOTANI[*3], HIROSHI KINUGAWA, YASUO INOUE,
KAZUMA HOSOKAWA AND TEISHIRO SEKI

Department of Public Health, Osaka University Medical School, Osaka

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SUMMARY

The properties of a tuberculostatic substance in sera of guinea pigs immunized with heat-killed *Mycobacterium tuberculosis* have been studied by an improved slide culture method. By this method it was possible to measure accurately the growth of tubercle bacilli in a media containing a high concentration of serum. The tuberculostatic substance in immune sera is not-dialysable, is resistant to heating at 65°C, but is inactivated when heated at 70°C, for one hour at a neutral pH. It is precipitated by 1.6 M $(NH_4)_2SO_4$. It is removed from immune sera by absorption with tubercle bacillus, but not with either *Staphylococcus aureus* or *Escherichia coli*. Treatment of immune sera with neither tuberculoprotein nor bentnite influences its tuberculostatic activity. The tuberculostatic activity of immune sera is suppressed by citrate or phosphate and this suppression is reversed by $MgSO_4$.

The results obtained provide strong evidence that the tuberculostatic substance in immune guinea pig sera is an antibody and is distinct from non-specific antibacterial substances, which increased in concentration as a result of immunization.

INTRODUCTION

In a previous study (Kotani *et al.*, 1956b) it was demonstrated, by the slide culture method specially improved to measure accurately the growth of tubercle bacillus in a media containing a high concentration of serum (Kotani *et al.*, 1956a), that sera taken from guinea pigs immunized with heat-killed tubercle bacillus exhibited a limited, but definite growth inhibiting activity against tubercle bacillus (tuberculostatic activity). It has been also shown that the tuberculostatic activity of immune sera was lost by heating the sera at 56°C for 30 minutes and restored by addition of fresh, normal guinea pig serum as complement to the inactivated sera.

The present paper deals with the results of the experiments which have been undertaken to characterize the tuberculostatic substance in immune sera to see whether the active substance is a specific antibody.

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*3 Present address: Department of Bacteriology, Nara Medical College, Kashiwara, Nara.

MATERIALS AND METHODS

1. *Method of slide culture*

The strain of tubercle bacillus used as a test organism, the culture bottles and the procedures for preparation of single bacterial cell suspensions and of bacterial films were all described in detail in the previous report (Kotani *el al.*, 1956a).

The tuberculostatic activity of immune sera was determined by comparing the extent of growth of tubercle bacillus in the immune sera to be tested, with that in control normal sera in the following manner: 1) The media used contained indicated amounts of test immune or normal serum specimens, a 0.05 M (a final concentration) glycylglycine-NaOH buffer, pH 7.3, and 2.0 or 2.5 ml of fresh, normal guinea pig serum as complement in a total amount of 5 ml. 2.5 ml aliquots of this medium were distributed in culture bottles containing a glass slide covered with a bacterial film prepared by using either silicic acid jelly or rabbit plasma. 2) The culture bottles were incubated at 37°C in air containing 5 per cent CO_2. After 3 days incubation, the slides were removed, fixed by immersion in 10 per cent formalin solution and stained by the Ziehl-Neelsen method. 3) Eleven microscopic fields were selected at equal intervals along the long axis of the stained bacterial films and examined. The number of tubercle bacillus in each of the microcolonies examined was counted as exactly as possible. The average number per microcolony per bacterial film was then calculated and expressed as a log value (in general 100 or more colonies were examined microscopically). Two slides were examined per test serum, the figures obtained as described above were averaged and this average value was taken to represent the extent of growth of tubercle bacilli in the test serum medium.

As a rule, more than two specimens of both immune and normal sera were used in each set of experiments.

2. *Standard used for the presence or absence of tuberculostatic activity in the test immune sera*

The standard adopted by Kotani *et al.* (1956b) in the previous study was followed, except that a correction was made for the possible presence of small clumps* of bacilli in the bacterial suspensions used as inoculum, by substracting the log of an average number of bacilli per viable unit in the bacterial films before cultivation from each of the figures representing the extent of growth of the bacilli in the test immune and normal sera.

The ratio of the extent of growth of tubercle bacilli in an immune serum to that in a normal serum was calculated and if this ratio X 100 was less than 90, the tuberculostatic activity of the test immune serum was defined as positive. In accompanying tables the figures obtained in this manner are presented as "relative values".

As reported in the previous paper (Kotani *et al.*, 1956b), the tuberculostatic activity of immune sera is due to the combined action of both an immune factor and complement, therefore it persists for only one day on incubation as a result of inactivation of complement. This is why a difference of 10 per cent between the log of the average bacterial number per microcolony grown in immune serum and that in normal serum, though small, must be considered as significant. It will be seen in a later section that the standard adopted in the present and previous studies is sufficiently reliable and by no means unreasonable in practice.

3. *The preparation of tuberculoprotein used as an antigen in complement fixation tests and as an absorbing agent*

This was prepared from an unheated culture filtrate of human type tubercle bacillus (strain Aoyama B) cultured in Sauton's medium for 8 weeks, according to the method of

* It is not always easy, especially on hot days in summer season, to get a tubercle bacillus suspension exclusively consisting of single cells.

Seibert and Glenn (1941). The details of the fractionation procedures used have been reported by Kotani *et al.* (1953).

4. *Immunization of guinea pigs*

In most experiments, use was made of immunizing antigen, prepared by suspending a 2 to 3 week old tubercle bacillus (strain Kamiike) grown on Oka-Katakura's egg yolk medium (malachite green was omitted) in liquid paraffin at a concentration of 20 mg/ml and by heating the suspension at 70°C for 2 hours. The suspension of the bacillus (strain H37Rv) in a mixture of liquid paraffin and dehydrated lanolin (3:1) at a concentration of 50 mg/ml, sterilized at 100°C for 30 minutes, was also used in some experiments. Male and female guinea pigs, weighing about 500 g, were infected in the lateral portion of the thigh with either 0.25 ml of the former antigen on both sides, or with 0.2 ml of the latter antigen on one side. The immunized animals were used in experiments more than 2 weeks after the injection. If necessary they were reimmunized in a similar manner.

RESULTS

In the experiments described in the following pages, both immune and normal serum specimens to be tested were subjected to similar treatments. The tuberculostatic activity of the non-treated and treated serum specimens was determined by comparing the growth of tubercle bacillus in these immune serum specimens with that in the corresponding normal ones, as described in the previous section.

1. *Heat stability of the tuberculostatic substance in immune sera*

Aliquots of sera were exposed to temperatures of 65°C and 70°C for one hour in the presence of glycylglycine-NaOH buffer (pH 7.2, 0.1 M in a final concentration) and the tuberculostatic activity, determined by the dilution method, of the heated immune serum specimens was compared with that of the non-heated, control sera.

Table 1. Heat stability of the substance in immune serum

Materials	Growth of tubercle bacillus (actual value*1)							Growth of tubercle bacillus in immune serum (relative value*2)			
	Normal serum			Immune serum							
	1:4*3	1:32	Mean	1:4	1:8	1:16	1:32	1:4	1:8	1:16	1:32
Non-treated serum	0.561	0.547	0.554	0.447	0.457	0.460	0.499	81	82	83	90
Heated serum at 60°C for 1 hour	0.552	0.521	0.537	0.475	0.456	0.469	0.507	88	85	87	94
at 70°C for 1 hour	0.560	0.515	0.538	0.545	0.537	0.519	0.524	102	100	96	98

*1 (The log of the average becterial number per microcolony grown in test serum for 3 days) — (the log of the average bacterial number per viable unit in a becterial film before cultivation).

*2 The values calculated as percentage of the extent of growth in the corresponding normal serum specimens.

*3 The final dilution of test serum.

It can be seen from the results given in Table 1 that the substance responsible for the tuberculostatic activity of immune serum withstands heating at 65°C for one hour, but is completely inactivated by heating at 70°C for the same

period.

2. *Effect of dialysis on the tuberculostatic activity of immune sera*

Aliquots of test sera were dialysed through cellophane membranes against a large quantity of physiological saline for 2 days at room temperature under aseptic precautions. The tuberculostatic activity of non-treated and dialysed immune serum specimens was determined.

Table 2. Dialysability of the tuberculostatic substance in immune serum

Materials	Growth of tubercle bacillus (relative value)			
	1 : 2*1	1 : 4	1 : 8	1 : 16
Not-dialysed serum	80	—*2	85	87
Dialysed serum	81	74	89	99

*1 Tne final dilution of test materials.
*2 The bacterial film separated off.

The results presented in Table 2 show that dialysis did not effect the tuberculostatic activity of immune serum and that the active substance does not dialyse through cellophane.

3. *Fractionation of immune serum with ammonium sulphate*

A part (about 8 ml) of test serum specimens was set aside as an untreated control and the remaining 40 ml was fractionated with ammonium sulphate. The precipitate which

Table 3. Fractionation of immune serum with ammonium sulphate

Materials		Growth of tubercle bacillus (relative value)
Starting serum	1:2.5*1	78
Fraction precipitated at 1.6 M $(NH_4)_2SO_4$ concentration	1 : 1	78
Fraction soluble at 1.6 M and precipitated at 2.1 M $(NH_4)_2SO_4$ concentration		103
Fraction soluble at 2.1 M and precipitated at 2.8 M $(NH_4)_2SO_4$ concentration		99

Materials	Growth of tubercle bacillus (relative value)				Titers of anti-tuberculoprotein antibody*2
	1:2.5*1	1:5	1:10	1:20	
Starting serum	—*3	87	94	97	1:40
Fraction precipitated at 1.6 M $(NH_4)_2SO_4$ concentration	60	71	78	102	1:2.5

*1 The final dilution of test materials.
*2 Measured by complement fixation test.
*3 The bacterial film separated off.

formed at the concentration levels indicated in Table 3 were collected by centrifugation. Each of the precipitates was dissolved in about 15 ml of saline and the solutions were dialysed against several changes of saline for 3 days. The dialysed solutions (concentrated to 16 ml by pervaporation) of each of the fractions were sterilized by filtration through a Shofu's bacterial filter (Berkefeld type) and their tuberculostatic activity was compared with that of untreated specimens, as shown in the Table.

The results are presented in Table 3. It can be seen that the fraction precipitated at 1.6 M ammonium sulphate was the only one manifesting tuberculostatic activity. Moreover, this fraction was rather more active than whole serum. In the column on the right of the lower part of the Table, the titers of the antibody to tuberculoprotein which were determined by the complement fixation test by the methed of Kotani *et al.* (1956b), of the unfractionated, control serum specimen and the fraction precipitated at 1.6 M ammonium sulphate are presented. There is no correlation between the titer of the anti-tuberculoprotein antibody and the strength of the tuberculostatic activity.

4. *Absorption of the tuberculostatic substance in immune serum with homologous and heterologous bacteria*

A living cell suspension of tubercle bacilli in 0.04 per cent Tween 80—0.4 per cent bovine plasma albumin solution and heat-killed (65°C for one hour) cell suspension of *Esch. coli* (strain UKT-B) and *Staph. aureus* (strain Terajima) in saline were used as homologous and heterologous absorbing agents, respectively. Tubercle bacillus was cultivated on Oka-Katakura's egg yolk medium for 2 weeks and the other two organisms were grown on nutrient agar medium for 18 hours.

To 1.8 ml aliquots of test serum specimens were added 0.45 ml of each bacterial cell suspension prepared as above (10 mg and 2.5 mg wet weight/ml) and 0.04 per cent Tween 80—0.4 per cent albumin solution or saline in the controls. The mixture were incubated at 37°C for 2 hours and left at 4°C for 2 days. They were centrifuged and the tuberculostatic activity of the supernatants from immune serum specimens was tested.

Table 4. Absorption of the tuberculostatic substance in immune serum with homologous and heterologous bacteria

Materials		Growth of tubercle bacillus (relative value)	
		Serum No. 1	Serum No. 2
Serum treated with:			
Physiological saline		81	89
0.04% Tween 80–0.4% plasma albumin		81	85
Living cells of *Myc. tuberculosis* (strain H37Rv)	2.5 mg*	96	106
	0.63 mg	97	98
Heat-killed cells of *Esch. coli* (strain UKT-B)	2.5 mg	86	89
	0.65 mg	Not done	86
Heat-killed cells of *Staph. aureus* (strain Terajima)	2.5 mg	87	89
	0.63 mg	Not done	83

* Wet weight of bacteria used as absorbing agents per ml of test serum. The final dilution of test serum used was 1 : 2.

As shown in Table 4, the tuberculostatic activity of immune sera disappeared after absorption of the sera with living cells of tubercle bacillus, while both *Staph. aureus* and *Esch. coli* did not remove the activity. The active substance was shown to have a specific affinity for the cells of tubercle bacillus.

5. *Attempts to absorb the tuberculostatic substance in immune serum with tuberculoprotein*

A physiological saline solution (4 mg/ml) of tuberculoprotein was used as an absorbing agent in this experiment. Test serum specimens were treated with serial four-fold diltutions of this tuberculoprotein solution as in the preceding experiment and assayed for their tuberculostatic activity.

Table 5. Attempts to absorption of the tuberculostatic substance in immune serum witn tuberculoprotein

Materials		Growth of tubercle bacillus (relative value)	
		Serum No. 1	Serum No. 2
Serum treated with :			
Physiological saline		84	85
Tuberculoprotein	1 mg*	89	79
//	1×4^{-1} mg	88	79
//	1×4^{-2} mg	89	82
//	1×4^{-3} mg	81	80
//	1×4^{-4} mg	78	83
//	1×4^{-5} mg	86	76

*Weight of tuberculoprotein per ml of test serum
The final dilution of test serum was 1 : 2.5.

It can be seen from the results presented in Table 5 that absorption of immune sera with tuberculoprotein did not influence their tuberculostatic activity, at least under the experimental conditions adopted.

6. *Inability of bentnite to absorb the tuberculostatic substance in immune serum*

0.6 ml of a 6 per cent bentnite suspension in saline or of alone saline as a control, was mixed with 2.4 ml of test serum specimens. After incubation with occasional agitation for 30 minutes at 37°C, they were centrifuged and the supernatants were sterilized by filtration through a membrane filter Co 5 (Membranefilter-Gesellshaft, Sartorius-Werke A. G.). The tuberculostatic activity of the bentnite-treated immune serum specimens was compared with that of the saline-treated, control ones.

Table 6. Inability of bentnite to absorp the tuberculostatic substance in immune serum

Materials	Growth of tubercle bacillus (relative value)	
	Serum No. 1	Serum No. 2
Serum treated with :		
Physiological saline	76	—*
Bentnite	85	83

* The bacterial film separated off.
The final dilution of test serm was 1 : 2.5.

As shown in Table 6, definite tuberculostatic activity can be seen in the bentnite-treated immune sera. In consideration of the powerful absorbing capacity of bentnite for lysozyme or lysozyme-like substances, it can be concluded that the substance responsible for the tuberculostatic activity of immune serum, observed in the present study, is distinct from lysozyme or similar substances.

7. *Inactivation with citrate or phosphate and reactivation with magnesium sulphate of the tuberculostatic activity of immune serum*

As indicated in Table 7, various quantities of a KH_2PO_4–Na_2HPO_4 buffer (pH 7.2) or citrate solution were added to test serum specimens with or without magnesium sulphate and the tuberculostatic activity of these immune serum specimens was tested.

Table 7. Inactivation with phosphate and citrate and reactivation with ammonium sulphate of the tuberculostatic activity of immune serum

Materials	Growth of tubercle bacillus (relative value)
Serum added with :	
None	81
0.01 M* KH_2PO_4–Na_2HPO_4 buffer (pH 7.2)	98
0.005 M* KH_2PO_4–Na_2HPO_4 buffer (pH 7.2)	103
0.001 M* KH_2PO_4–Na_2HPO_4 buffer (pH 7.2)	104
0.0005 M* KH_2PO_4–Na_2HPO_4 buffer (pH 7.2)	89
None	82
0.025 M $Na_3C_6H_5O_7$	99
0.0125 M $Na_3C_6H_5O_7$	102
None	87
0.0067 M KH_2PO_4–Na_2HPO_4 buffer alone	102
0.0067 M KH_2PO_4–Na_2HPO_4 buffer +0.01 M $MgSO_4$	81
None	82
0.025 M $Na_3C_6H_5O_7$ alone	99
0.025 M $Na_3C_6H_5O_7$ +0.0375 M $MgSO_4$	85

* Final concentration.
The final dilution of test serum was 1:2.

It can be seen that the addition of phosphate or citrate to immune sera suppresses their tuberculostatic activity and that the addition of magnesium sulphate reversed this inactivation. The inactivating effect of phosphate and citrate is probably due to the withdrawal of magnesium ions from the test serum.

8. *Validity of the standard adopted for the presence or absence of the tuberculostatic activity of test immune serum*

In Table 8 the data collected from the protocols of the previous and present studies are presented in summary. In all of these experiments, use was made of silicic acid jelly film and glycylglycine-NaOH buffer, the final dilution of test serum specimens was two- or three-fold and the content of fresh serum as complement in reaction mixture are 40 or 50 per cent.

Table 8. Validity of the standard adopted for the presence or absence of the tuberculostatic activity of immune serum (Summary of the data obtained in the present and previous studies)

Experiment No.	Growth of tubercle bacillus (relative value)		Experiment No.	Growth of tubercle bacillus (relative value)	
	Immune sera	Normal sera		Immune sera	Normal sera
1	68	100	9	68	100
	68	100		85	100
	82		10	64	99
2	78	100		81	100
	78			82	101
3	71	100	11	82	99
	84			85	102
4	68	100	12	100	97
	85			103	103
	100		13	80	100
5	86	99	14	78	100
	88	100	15	84	100
	89	101	16	81	100
	90		17	59	98
6	73	100		73	99
	94	100		81	99
7	73	99		82	99
	78	99		93	106
	80	102	18	76	99
	85			<83	101
	87		19	79	100
	87			84	100
	91		20	88	100
	96		21	88	100
	98				
8	87	99			
	90	101			

The final dilution of test sera was 1 : 2 to 1 : 3.

It can be understood that the data shown in this Table that fluctuations of the extent of the growth of tubercle bacillus in normal serum specimens are surprisingly small in any one experiment and that the standard adopted for the presence or absence of the tuberculostatic activity of immune serum specimens, on the whole, is reliable and is by no means unreasonable in practice.

DISCUSSION

The problem of whether serum taken from the infected or immunized men and animals has any tuberculostatic activity, has long been the subject of study by many investigators. However no unanimous and definite conclusions have been reached (Rich, 1951)*.

In the previous study (Kotani *et al.*, 1956b), the authors have reported that immune guinea pig serum exhibited a definite tuberculostatic activity under

* Hubáček and Málek (1959) have reported a similar study on factors inhibiting growth of *Mycobacteria* in the blood and serum of immunized animals. However, their results and conclusion on the nature of the active factors are differed from those of the present authors in various points.

certain experimental conditions. The authors discussed the possible reasons for the failure of the former workers to obtain any decisive evidence of the tuberculostatic activity of immune serum. It has been also pointed out that the validity of the positive results, except those of Emmart and Seibert (1945) and Myrvik and Weiser (1951), also is doubtful in the light of results obtained in the author's experiments.

The studies presented in the present paper have confirmed the results reported in the previous paper and, furthermore, have clearly demonstrated that the tuberculostatic substance in sera of guinea pigs immunized with heat-killed tubercle bacilli, the activity of which can be observed by an improved slide culture method, is entirely different from possible, non-specific antituberculous substances (Myrvik and Weiser, 1951), properdin (Pillemer *et al.*, 1954) and others, which might non-specifically increase as a result of immunization procedures. The active substance is in all probability an antibody of some kind, as suggested by Emmart and Seibert (1945).

At present, it is not yet clear against which components of tubercle bacillus the tuberculostatic antibody is directed. In previous studies (Kotani *et al.*, 1953; 1956b) it has been reported that the tuberculostatic activity of whole blood taken from guinea pigs infected with virulent tubercle bacilli was abolished by addition of appropriate amounts of tuberculoprotein and that sera of guinea pigs immunized with tuberculoprotein in water in an oil emulsion exhibited a definite tuberculostatic activity. These results suggested that the tuberculostatic antibody might be produced against bacterial components corresponding to the tuberculoprotein. However, the results of the present investigation, contrary to expectation, indicated that the tuberculostatic activity could not be removed from immune sera by absorption with the amounts of tuberculoprotein used. Although various possibilities can be considered to explain the discrepancy between the results of the present and previous studies, further experiments are required to elucidate the antigen which evoked the tuberculostatic antibody.

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