STUDIES ON ANTITUBERCULOUS FACTORS IN THE LOW MOLECULAR FRACTIONS OF THE SERA OF VARIOUS ANIMAL SPECIES

REPORT I. CRUDE MATERIALS AND THEIR TUBERCULOSTATIC EFFECTS

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Introduction

From our laboratory, some papers[1-5] were reported concerning the role of humoral factors in the native and acquired resistance of animals to tuberculosis. And it has been demonstrated that the low molecular fraction (able to pass through a cellophane membrane) of body fluids of rabbits was inhibitory to the growth of virulent tubercle bacilli *in vivo*, and not inhibitory to that of non-pathogenic mycobacteria. The high molecular fraction (unable to pass through a cellophane membrane) of body fluids were promoting to virulent tubercle bacilli. It was also proved that the growth of virulent tubercle bacilli *in vivo* was completely inhibited in the body fluids of immunized rabbits. It has also been found that there were several substances having growth-inhibitory power to tubercle bacilli in human urine and in the low molecular portions of various organ extracts of rabbits. And it has been demonstrated that these active substances are composed of some amino acids, polypeptides and organic acids.

Subsequent investigations dealing with the tuberculostatic factors of the low molecular fractions in the serum of various animal species and their physico-chemical nature will be reported in this paper.

Materials and Methods

Preparation of materials

Crude materials of the low molecular fraction in the serum of various animal species, i.e., human, bovine, rabbit, goat, dog, cat, horse and guinea pig were prepared as follows.

Fresh serum was dialyzed through a cellophane membrane (for dialysis No. 300)

against ten or fifteenfold of sterile destilled water at 4°C for 72 hours. The outer fluid produced by dialyzation was concentrated under reduced pressure at 45°C in temperature to dryness, and was weighed.

The dried materials looked like milky white and contained crystals, and were viscous and deliquescent. The color of the dried materials easily changed to yellow at about 60°C. The pH of the dried materials changed from 6.6～7.0 to 8.6～8.8 by concentration. These dried materials were dissolved in 1/16～1/32 volume of starting serum with distilled water and were adjusted to pH 7.0.

Test for tuberculostatic activity.

The slide culture method was used.

Kirchner's medium with 10% goat serum containing the serially diluted crude materials, which had been sterilized at 100°C for 30 min. were prepared. Each one of the slides, smeared with the test bacteria (H37Rv, BCG, avian type Cho-Kyo strain of tubercle bacilli, Mycobacterium 607, respectively) by using the benzine method[6], was immersed into each one tube of the medium. Tubes were incubated for ten days at 37°C.

The degree of growth of the bacilli is designated as follows.

卅 : Colonies adhere to each other, and there are no isolated colonies. (observable by weak magnification)

廾 : Very good proliferation, but there are isolated colonies. (ibid.)

＋ : Good proliferation. (seen only by oil immersion)

－ : No growth.

In order to examine the influence of hemolysis on the tuberculostatic activity of serum, the outer fluid was collected by dialyzing hemolyzed red cell extract without serum and its tuberculostatic activity was tested. No tuberculostatic activity was noted.

Immunization

Eight rabbits weighing 2.5 kg-3.0 kg were divided into four groups. One group was inoculated intramuscularly with 100 mg of heat-killed H37Rv strain suspensed in paraffin oil twice at two weeks intervals. Three groups were inoculated once intramuscularly with 1 mg, 5 mg and 50 mg of living BCG, respectively. Three weeks after the last inoculation, the rabbits showing tuberculin-positive reaction were used for the experiment.

Results

1. Experiment concerning inhibitory activity of crude materials on the growth of H37Rv strain.

The tuberculostatic activity of crude materials in the serum of various animal species was tested. The results are shown in Table 1. Inhibitory activities in

Table 1. The effect of inhibitory activity of crude materials in the serum of various animal species on the growth of H37Rv strain.

Animal species	Number of cases	pH	Concentration of crude material						
			×16	×8	×4	×2	×1*	×½	×¼
Human	3	8.6	—	—	—	—	++	++	++
Bovine	8	8.6	—	—	—	+	++	++	++
Rabbit	5	8.6		—	—	—	+	++	++
Goat	2	8.6	—	—	—	++	++	++	++
Dog	4	8.6	—	—	—	++	++	++	++
Cat	2	8.6	—	—	—	++	++	++	++
Horse	2	8.6	—	—	—	++	++	++	++
Guinea pig	2	8.6		—	—	++	++	++	++

Control : ++
Slide culture using H37Rv strain in Kirchner's medium for ten days.
* ×1 means the same concentration as in the corresponding serum, and according to the increase of number the materials are concentrated.

all materials tested were noted, and the activities seemed to be a little more powerful in human and rabbits than in others. But there was no remarkable difference among either the same or different animal species.

2. Experiment concerning inhibitory activity of crude materials from immunized rabbits on the growth of H37Rv strain.

The tuberculostatic activity of crude materials in the serum of immunized rabbits was tested and compared with normal rabbits. As shown in Table 2, no significant difference between immunized and normal rabbit was found.

Table 2. The effect of inhibitory activity of crude materials in the serum of immunized rabbits on the growth of H37Rv strain.

Method of immunization	Concentration of crude material					
	×16	×8	×4	×2	×1	×½
1 mg living BCG, once.	—	—	—	—	++	++
5 mg living BCG, once.	—	—	—	++	++	++
50 mg living BCG, once.	—	—	—	—	++	++
100 mg heat killed H37Rv, twice.	—	—	—	++	++	++
No immunization (control.)	—	—	—	—	++	++

Control : ++
Slide culture using H37Rv strain in Kirchner's medium for ten days.

3. Experiment concerning inhibitory activity of crude materials on various mycobacteria.

Various strains of mycobacteria were cultured by the slide culture method,

and inhibitory activity of crude materials in the serum of several animals, (bovine, rabbits and others), was examined. The results are shown in Table 3. Inhibitory activity was observed in all materials tested on the growth of virulent tubercle bacilli, but not on non-pathogenic mycobacteria, (Myco. 607) indefinite to the avian type of tubercle bacilli.

Table 3. The effect of inhibitory activity of crude materials in the serum of various animals on the growth of various mycobacteria.

Animal species	Strain	Number of case	Concentration of crude material					
			×16	×8	×4	×2	×1	×½
Bovine	H37Rv	3	—	—	—	+	++	++
	BCG	3	—	—	—	++	++	++
	Avian Cho-Kyo	2	— —	— —	— ++	— ++	++ ++	++ ++
	M. 607	2	++	++	++	++	++	++
Rabbit	H37Rv	2		—	—	—	+	++
	BCG	2		—	—	—	+	++
	Avian Cho-Kyo	2		— —	— ++	— ++	++ ++	++ ++
	M. 607	2		++	++	++	++	++
Dog	H37Rv	2	—	—	—	++	++	++
	BCG	2	—	—	—	++	++	++

Control : ++
Slide culture in Kirchner's medium for ten days.

4. The solubility of crude materials and their growth inhibiting effects.

For the purpose of inferring the chemical properties of tuberculostatic substance, solubility in water and in several organic solvents of crude materials were examined. As the organic solvents, methanol, ethanol, buthanol, ethyl ether, acetone and benzene were used. The crude dried materials were added to a large quantity of solvent, and its soluble and insoluble portions were separated. After these portions were evaporated to dryness for removing the solvents, the tuberculostatic activity was tested. The results of the bovine material are shown in Table 4.

The tuberculostatic substances were soluble in water and in methanol, but insoluble in buthanol, ethyl ether, acetone and benzene. The tuberculostatic activities were noted both in soluble and insoluble portions when ethanol was used as a solvent.

It seems that methanol extraction is the most effective way of the purification of crude materials. And the inhibitory activity on the growth of BCG were same as on H37Rv.

Table 4. The solubility of crude materials in the bovine serum and their inhibitory activities.

Solvent	Rate of solubility	pH	Concentration of material						Minimum concentration enough to inhibit the growth
			×32	×16	×8	×4	×2	×1	
Water, Soluble	100%	8.6	—	—	—	—	±	++	33.7 mg/dl
Methanol, Soluble	48.6%	7.6	—	—	—	—	++	++	16.4
Insoluble	51.4%	8.2	±	++	++	++	++	++	—
Ethanol, Soluble	11.5%	7.4	—	+	++	++	++	++	31.0
Insoluble	88.5%	8.4	—	—	—	+	++	++	29.8～59.8
Buthanol, Soluble		7.2	++	++	++	++	++	++	
Insoluble		8.6	—	—	—	—	+	++	
Ethyl-ether, Soluble	15.4%	6.6	++	++	++	++	++	++	—
Insoluble	84.6%	8.6	—	—	—	—	+	++	28.5
Acetone, Soluble	11.8%	6.6	++	++	++	++	++	++	
Insoluble	88.2%	8.6	—	—	—	—	±	++	
Benzene, Soluble		6.6	++	++	++	++	++	++	
Insoluble		8.6	—	—	—	—	±	++	

Control : ++
Slide culture using H37Rv strain in Kirchner's medium for ten days.

The solubility of crude materials from the sera of human, goats and cats were investigated by the same way, and their inhibitory activities were tested. In these cases, the active substances were soluble in methanol, insoluble in ethanol, acetone, and ethyl ether. The results of human material are shown in Table 5.

The solubility in organic solvents of crude materials of goat's and cat's serum and their inhibitory activities were similar to those of bovine serum.

Table 5. The solubility of crude materials in the human serum and their inhibitory activities.

Solvent	pH	Concentration of material					
		×16	×8	4×	2×	1×	×½
Water, Soluble	8.6	—	—	—	—	+	++
Methanol, Soluble	7.6	—	—	—	+	++	++
Insoluble	8.2	++	++	++	++	++	++
Ethanol, Soluble	7.4	++	++	++	++	++	++
Insoluble	8.4	—	—	—	+	++	++
Ethyl-ether, Soluble	7.0	++	++	++	++	++	++
Insoluble	8.1	—	—	—	+	++	++
Acetone, Soluble	7.0	—	++	++	++	++	++
Insoluble	8.6	—	—	—	—	++	++

Control : ++
Slide culture using H37Rv strain in Kirchner's medium for ten days.

5. The stability against heat and hydrolysis

Bovine crude material was used.

The crude materials were devided into 5 groups and after being neutralized, they were heated as follows.

(1) No heating (sterilization through Seitz's filter)
(2) Heating at 100°C for 30 min.
(3) Heating at 100°C for 60 min.
(4) Heating at 120°C for 30 min.
(5) Heating at 120°C for 60 min.

Table 6 shows the results of the inhibitory activity of heated materials. No influence of heating were noted. It was certain that the inhibitory activity of neutralized crude materials was considerably heat stable.

The iufluence of hydrolysis on the tuberculostatic activity was examined. The crude material was hydrolysed with 6N HCl at 100°C for 15 hours, and the tuberculostatic activity was compared with that of the material before hydrolysis.

Table 6. The stability against heating and hydrolysis of crude materials and their inhibitory activities.

Treatment	Concentration of material					
	×16	×8	×4	×2	×1	×½
Heating at 100°C for 30 min.	—	—	—	+	++	++
Heating at 100°C for 60 min.	—	—	—	+	++	++
Heating at 120°C for 30 min.	—	—	—	+	++	++
Heating at 120°C for 60 min.	—	—	—	+	++	++
No heating	—	—	—	+	++	++
Before hydrolysis	—	—	—	+	++	++
After hydrolysis	—	—	—	+	++	++

Control : ++
Hydrolysis is performed at 100°C for 15 hrs. with 6 N HCl. Slide culture using H37Rv strain in Kirchner's medium for ten days.

6. Other chemical properties of crude materials.

Chemical qualitative analysis of crude materials was carried out. The results are as follows.

Biuret reaction; In all materials biuret reaction was always negative. We can use this method testing the completeness of dialysis.

Saturation of ammonium sulfate; No precipitation was noted.

Ninhydrine, Millon's, Sakaguchi's and Ehrlich's reactions; All were positive; therefore, it may be inferred that there are many chemical substances mixed in the materials.

Discussion

Tsuji and his associates[1] have previously investigated the influence of body fluids of rabbits on the growth of various mycobacteria *in vivo* using the chamber method. They have found that the low molecular fraction (able to pass through a cellophane membrane) of body fluids of rabbits can inhibit the growth of virulent tubercle bacilli and has no influence on the growth of non-pathogenic mycobacteria. The present author have attempted to examine whether or not the growth inhibitory activity of the low molecular fraction of body fluids can be proved not only *in vivo* but also *in vitro.* The sera of various animal species were used as the original material of body fluid.

The outer fluid produced by dialysis of the serum through a cellophane membrane was concentrated and was tested on the growth of various mycobacteria.

It has been proved that the low molecular fraction in the serum of various animals (human, bovine, rabbits and etc.) could uniformly inhibit the growth of virulent tubercle bacilli and the inhibitory effect on the avian strain of tubercle bacilli was indefinite. Myco. 607 was always not inhibited *in vitro.* These results were the same as those obtained by the chamber method *in vivo.*

As to the physico-chemical nature of these active substances, the solubility in water and in several organic solvents was examined. They were easily soluble in water and in methanol, but insoluble in buthanol, ethyl ether, acetone and benzene, and partially soluble in ethanol. The stability against heating and hydrolysis was examined. These substances were stable and their activities did not decrease even with heating at 120°C for 60 min. or hydrolysis at 100°C for 15 hours with 6N HCl.

Reductive reaction and amino acid reaction were tested and were found to be positive. From these facts it may be said that the low molecular fraction dealt here is not a single substance but rather a mixture of some intermediate metabolites.

Antituberculous agents found in normal animal bodies have been reported by some investigators, e.g., urine factor by Björnesjö[7-14], fatty acids of the lung by Patnode[15], basic peptide, spermine, spermidine and fatty acids (lactic acid etc.) by Dubos[16-21] and so forth.

In our laboratory, Oshima[22] reported a low molecular autituberculous substance in the human urine, and Nakashima[23] found a similar low molecular substance in the organ extracts of rabbits. The properies of the low molecular antituberculous substances obtained in the present study are similar to those described above. Therefore, it may be reasonable to say that these active substances are released from the same unknown foci to the body fluid as the metabolic products.

It may be of interest that the activities of the low molecular substances of

immunized sera are quite similar to those of the normal non-immunized sera.

While, it has already been confirmed in our laboratory that the tuberculostatic activity of the body fluids increased *in vitro* after immunization when the ring method was used[4]. Therefore, it may be certain that the increase of antituberculous activity appearing in the body fluids after immunization are due to the action of high molecular fraction (perhaps protein).

These low molecular antituberculous substances may have some significant role in the native resistance to tuberculosis.

Summary

1) The tuberculostatic activity of the low molecular fraction (able to pass through a cellophane membrane) in the serum of various animal species (human, bovine, rabbits, goats, dogs, cats, horses and guinea pigs) was observed. No distinct difference among the various animal species was existed.

2) The antituberculous substances did not possess the inhibitory activity on the growth of Mycobacterium 607 and the effect on the avian strain (Cho-Kyo strain) of tubercle bacilli was indefinite.

3) The substances are soluble in water and in methanol, insoluble in buthanol, ethyl ether, acetone and benzene, and soluble partially in ethanol.

4) The substances are stable against heating at 120°C for 60 min. and against hydrolysis at 100°C for 15 hours with 6N HCl.

5) The activity of these substances was not changed by immunization of the rabbit.

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