Study on the Susceptibility of Rats to Various Strains of Mycobacteria

Report III. The Humoral Defensive Power of Rats Against Mycobacteria

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INTRODUCTION

In the previous report, it was demonstrated that the albino rat has more native resistance to various strains of virulent tubercle bacilli than the rabbit, the guinea pig and also the harmster. Although bacilli which are almost fatal to susceptible animals were capable, when inoculated in albino rats, of multiplying in the organs, especially in the lung, in the earlier stages of infection, they later ceased to grow for a while and after 4 weeks they decreased in number when tested by quantitative cultivation. None of rats died from infection.

In regard to this strong resistance of rats to tuberculosis, the absence of tuberculin allergy in the animal is in general thought to play a very important role. However, the true mechanism is not clear.

Tsuji and associates[1)2)3)4)5)] reported various interesting results concerning the role of humoral factors in the natural and acquired resistance of rabbits to tuberculosis. Some of their important findings are as follows ; Virulent and attenuated tubercle bacilli were capable of growth in the body fluid completely separated from cells *in vivo*. An analytical experiment indicated that the low molecular factor of body fluid had a growth-inhibiting action on these bacilli and the high molecular factors of body fluid inhibited this action of the low molecular factor and consequently the bacilli could multiply in total body fluid of the rabbit.

Humoral defensive power, therefore, must be valued in addition to the cellular defensive power which may be also significant in the mechanism of natural resistance of animals to tuberculosis.

In this paper, experimants related to the role of the humoral factor in native resistance to tuberculosis of the albino rat will be reported.

MATERIALS AND METHODS

Wistar albino rats weighing 240-300 gm. and bred in our laboratory for one

month before the beginning of the experiment and confirmed to be completely healthy, were used.

Cultures : Preserved laboratory strains

Virulent human type H37Rv strain, bovine type RM strain, BCG strain, resistant variant of H37Rv to 50γ isoniazid (catalase negative), H37Ra strain, avian Cho-Kyo strain, non-pathogenic 607 strain and non-pathogenic smegma strain were used.

All strains had been preserved continuously, and 14-21 day's colony on Sauton's media or 7-10 day's colony on Kirchner's media were used for experiments.

(1) Chamber method

This method has been reported by the original writers[4)5)] in detail and the description of the method will be omitted here. By this method one can observe the growth of mycobacteria in the body fluid completely separated from cells *in vivo* (the O-chamber) and in the medium in which only the lower molecular fraction can act on the bacilli *in vivo* (the K-chamber).

However, a few points which are different from the description in the original paper will be noted here.

The size of the chamber used was naturally smaller than that used in the rabbit. Ordinarily a plastic plate of 1×1 cm. in size was used. Cultivation was usually continued to the 30th-40th day after implanting.

As a control experiment the following procedures were carried out.

1) Ordinary slide culture[6)7)] using the same bacillary suspension as the bacilli used in the chamber parallels the chamber-cultivation.

2) Several chambers using the same bacilli were implanted in the rabbit to make sure of the availability of the bacilli used.

(2) Air-tight cultivation method

This method has also been reported in detail by Tao.[8)] The technique of this method is as follows :

Blood is collected in a syringe containing ca. 5 ml. of sterile paraffin oil. Centrifugation of blood is carried out also under paraffin oil. This separated serum is poured into a test tube and 5 ml. of paraffin oil are added. In this undiluted serum a slide smeared with mycobacteria by the benzine method[6)9)], was cultured air-tight for about 3-7 days.

As a control experiment, the same serum was exposed to air for half a day and after that the same cultivation as described above using this serum was carried out.

(3) Slide culture method

Blood was obtained by heart puncture. Serum was separated by centrifugation. Modifying from the original slide culture method, a series of serum media diluted to 10-90% with 10 times concentrated Kirchner's liquid was made as shown in the Table below.

Using these serum media, slide cultures were performed.

	Serum	10 times Concentrated Kirchrer's liquid	Distilled water
10%	1	1	8
20%	2	1	7
30%	3	1	6
40%	4	1	5
50%	5	1	4
60%	6	1	3
70%	7	1	2
80%	8	1	1
90%	9	1	0

RESULTS

(I) Normal untreated rats

(1) Results obtained by the chamber method:

As shown in Table 1, the virulent H37Rv strain, the bovine RM strain, the attenuated INH-resistant variant of the H37Rv strain, the H37Ra strain, and the BCG strain apparently grew in the O-chambers implanted in rabbits, but they were hardly capable of multiplying in the K-chambers. Avian Cho-Kyo strain could grow in both the O- and K-chambers. These results agree completely with the results reported by the original writers. In rats, on the other hand,

Table 1. Growth of Bacilli in the Chamber Inserted into the Peritoneal Cavity of Rat.

Animal \ Strain / Chamber		H37Rv Strain	Bovine RM Strain	BCG Strain	H37Ra Strain	Resistant Variant of H37Rv Strain	Avian Type (Cho-kyo) Strain	Myc. Smeg.	Myc. 607
Rat	O-Chamber	−	−	+	−	−	+	−	−
	K-Chamber	−	−	−	−	−	−	−	+
Rabbit (Control animal)	O-Chamber	++	++	+	++	++	+	−	−
	K-Chamber	−	−	±	−	−	+	++	++

− No Proliferation
± Proliferation Not Decisive.
+ Proliferation Apparent.
++ Proliferation Very Remarkable.

most of the virulent and attenuated tubercle bacilli were unable to multiply at all in both the O- and K-chambers, except for the BCG strain and the avian strain. These latter two strains could grow in the O-chambers, though not in the K-chamber. These characteristics of the BCG and avian strains were observed also in hamsters by Tao.[8]

Smegma bacilli and Myco. 607 could grow only in the K-chamber and did not grow in the O-chamber in rabbits. Although in rats Myco. 607 was capable of growth in the K-chamber in spite of no growth in the O-chamber, smegma bacilli was unable to grow in either chamber.

It may be concluded that the body fluid of the rat is more unsuited for the *in vivo* growth of virulent and attenuated tubercle bacilli (except BCG strain) than that of the rabbit. And both low and high molecular fractions of body fluid of the rat inhibit these bacilli *in vivo*, though the high molecular fraction antagonizes the inhibiting action of the low molecular fraction of body fluid in rabbits.

This greater difficulty in growing of bacilli in body fluid of the rat may play a very important role in the fact that the rat was much more native resistance to these pathogens than the rabbit.

(2) Results obtained by the slide culture method

Table 2. The Growth of Bacilli in the Kirchner's Media Containing Various Concentrations of Serum

Per Cent of Serum(%) / Strain	10	20	30	40	50	60	70	80	90	Whole Serm	Incubation Period (days)
H37Rv Strain	++	++	++	++	++	+	−	−	−	−	7
Bovine Type RM Strain	++	++	++	++	++	±	−	−	−	−	7
H37Ra Strain	++	++	++	++	++	+	+	±	±	−	7
BCG Strain	++	+++	+++	++	++	++	+	±	±	±	7
Avian Type (Cho-Kyo) Strain	+	+	+	+	+	+	+	+	+	+	7
Myc. Smeg.	+	+	+	+	+	+	+	+	+	−	5
Myc. 607	++	++	++	++	++	++	++	++	++	++	3

As shown in Table 2, bacillary growth was seen easily in tubes containing from 10 to 50 per cent serum. Virulent H37Rv and bovine RM strains were restricted in their growth in media containing higher concentrations of serum than 70 per cent. Attenuated H37Ra and BCG strains could grow more easily than these virulent strains. The avian strain and non-pathogenic strains were capable of growth in almost 100 per cent serum.

These relations do not parallel the *in vivo* behaviour of these bacilli, and may be strongly influenced by *in vitro* conditions of cultivation. Therefore, it is clear that ordinary slide culture method is inadequate for testing the action of serum in *in vivo* relationships.

(3) Results obtained by the air-tight cultivation method results are shown in Table 3.

Table 3. Growth of Bacilli by Air-Tight Cultivation Method

Animal \ Strain		Human Type H37Rv Strain	Bovine RM Strain	BCG Strain	H37Ra Strain	Avian Type (Cho-kyo) Strain	Myc. Smeg.	Myc. 607
Rat	Air-Tight Cultivated	+	+	−	+	+	−	+
	Exposed to Air (Control)	−	−	−	−	+	−	+
Rabbit (Control Animal)	Air-Tight Cultivated	+	+	−	+	+	+	+
	Exposed to Air (Control)	−	−	±*	−	+	+	+

* Result was precarious

In the rabbit's serum used as a control, H37Rv, bovine RM, H37Ra, avian Cho-Kyo, non-pathogenic smegma bacilli, and Myco. 607 were capable of growth, when cultured under air-tight conditions. However, the former three strains were inhibited in their growth when cultured in media exposed to air. The latter three strains were unchanged in their growth under the latter conditions. The BCG strain was a unique strain which was incapable of growth both in air-tight and exposed media. These results agree well with those obtained by Tao.

In the rat's serum, however, contrary to results obtained by Tao in the hamster, H37Rv, bovine RM and H37Ra were capable of growth, when cultured under air-tight conditions. Cho-Kyo and Myco. 607 could also grow anaerobically, but not smegma bacilli.

In media exposed to air, all strains but Cho-Kyo and Myco. 607 strain were incapable of growth. These results are very similar to results obtained in the control rabbit's serum.

No significant difference of rat's serum from rabbit's serum was noted, though Tao[8] reported that in the hamster the H37Rv and bovine RM strain were restricted in their growth in air-tight cultivation and thus he assumed that a stronger inhibiting power of the hamster's serum than the rabbit's had been demonstrated by this particuler technique of cultivation. Therefore, it may be that the significance of the results obtained by this air-tight cultivation technique should be evaluated with care.

(II) Rats given cortisone

In order to determine the significance of the humoral factors in the increased growth of tubercle bacilli in the cortisone-treated animal, Tsuji and his associates[10)11)] performed *in vitro* and also *in vivo* experiments by the chamber technique. It was demonstrated in these experiments that virulent tubercle bacilli (H37Rv and bovine RM strain) grew apparently more rapidly and more vigorously in the body fluid completely separated from cells in the cortisone-treated rabbit *in vivo* than in the body fluid from the normal untreated rabbit. It was also observed by the particular *in vitro* experiment (the ring method[3)12)]) that serum collected from an animal treated with cortisone had a definite growth-stimulating action on tubercle bacilli.

It has been demonstrated in this paper that the body fluid of rats possesses a more powerful growth-inhibiting activity against these virulent tubercle bacilli *in vivo* than that of the rabbit, —— these bacilli are restricted in their growth even in the O-chamber implanted in the rat's peritoneal cavity in spite of apparent growth of bacilli in the same chamber implanted in the rabbit. It may be of interest to test, therefore, whether or not the body fluid of the rat given cortisone acquires some tuberculo-stimulating properties and bacilli can grow in the chamber.

Each of 10 albino rats weighing 240-300 g. had two O-chambers and two K-chambers implanted in the peritoneal cavity. Starting the next day, cortisone acetate (5 mg. per kg. per day) was subcutaneously administered every day until the 40th day. As control 1, five albino rats had the same chambers implanted as just described and were observed without cortisone administration. As control 2, two rabbits were had the same chambers and were observed without cortisone.

H37Rv, bovine RM, H37Ra, BCG and avian Cho-Kyo strain were used as test

Table 4. Growth of Bacilli in the Chamber Inserted into the Peritioneal Cavity of Cortisone-treated Rat.

Animal \ Strain			Human Type H37Rv Strain	Bouine Type RM Strain	H37Ra Strain	BCG Strain	Avian Type (Cho-kyo) Strain
Rat	Cortisone-treated	O-Chamber	−	−	−	+	+
		K-Chamber	−	−	−	−	−
	Untreated (Control)	O-Chamber	−	−	−	+	+
		K-Chamber	−	−	−	−	−
Rabbit (Control Animal)	Untreated (Control)	O-Chamber	++	++	++	+	+
		K-Chamber	−	−	−	±	+

bacteria.

Animals treated with cortisone lost weight to 1/3 of their original weight up to the 10th day, but after that there were almost no changes.

On the 41st day after the implanting, the animals were bled by heart puncture, and the chambers were removed.

As shown in Table 4, in the control rabbit the growth of bacilli in the O-chambers was confirmed, and therefore in was certain that the bacilli used were quite suitable for the experiments.

In the rats, the growth of bacilli in the chambers was uniform both in animals given cortisone and in those not treated. Except for weak multiplication of the BCG and avian strains in the O-chambers, no growth of virulent strains or of the H37Ra strain was noted in either the O- or K- chambers.

There was no marked change in the stimulating action of the body fluid of albino rats treated with cortisone on the growth of bacilli *in vivo*. Even if some changes had occurred, they might not be powerful enough to overcome the growth-inhibiting action of the rat's body fluid.

DISCUSSION

In the previous experiments, it was shown that, though virulent tubercle bacilli were capable of growth to some extent in the organs of albino rats, this multiplication ceased after a while, and none of the animals succumbed to the infection. This fact indicates that albino rats have much more native resistant to tubrculosis than rabbits, guinea pigs and also hamsters. In this paper, it has been shown by the chamber method that the body fluid of albino rats possesses a powerful inhibiting action on the growth of virulent tubercle bacilli *in vivo*, and besides that in addition to the low molecular factor of body fluid the high molecular fraction which acted to stimilate growth in rabbits was completely growth-inhibiting in rats.

It is necessary to add that this action of body fluids is by no means bactericidal. The chamber in which no bacillary growth was noted was taken out 30 days after implantation. The watch glass was immersed in Kirchner's medium, after being treated with 5% H_2SO_4, and was cultivated as a type of slide culture. After 7 days vigorous growth of smeared bacilli on the watch glass was noted. Therefore, in spite of no growth *in vivo*, it was certain that these bacilli had remained alive.

It may be certain, therefore, that this inhibiting property of the body fluid of rats on the growth of virulent tubercle bacilli *in vivo* may play a very important role in the strong native resistance of these animals to tuberculosis. This fact may be a quite new finding, in addition to the similar fact in hamsters

found by Tao[9], which may be helpful for the explanation of the mechanism of native resistance, combined with the sensitivity to tuberculin allergy of these animals.

In vitro experiments using the serum of albino rats, —— slide culture and hypoaerobic cultivation —— no significant results in the relationships between the growth of pathogens and the resistance of the host was obtained. These *in vitro* culture methods seem to be inadequate for the resolution of the problem of host-parasite relationship, though Tao found a somewhat reasonable relation between the growth of bacilli cultivated anaerobically and the resistance of hamsters to these parasites.

The enhancing effect of cortisone on the growth of virulent tubercle bacilli *in vivo* was not definite and rather insignificant in the rat, though it was very conspicuous in the rabbit. This may suggest that the change of body fluid affected by cortisone treatment may not be uniform according to the animal species used. And it might be possible to postulate that the ability of animals to form antibody may actually be related to the effect of cortisone on the body fluids of animals.

It is noteworthy that the BCG strain and also the avian strain has a particular property in the growth *in vivo* in the body fluid of the albino rat. These strains can multiply in the O-chamber. The same facts were noted in the hamster by Tao.

SUMMARY

In order to explain the mechanism of the high native resistance of albino rats to tuberculosis, growth-experiments *in vivo* using various strains of mycobacteria were carried out using the chamber method. Virulent tubercle bacilli (human and bovine) could not grow at all in the O- and K-chambers. These results are different from results obtained by experiments in rabbits, in which bacilli were capable of growth in the O-chamber, but not in the K-chamber. It may be also concluded that the high molecular factor of body fluid in rats is tuberculostatic, although the high molecular factor of rabbit's body fluid acts to stimulate-growth. The low molecular factor of rats is of course growth-inhibiting, as it is in rabbits. Therefore, it may be that this property of body fluid in rats plays a very important role in the high resistance to tuberculosis of this animal.

In vitro experiment including anaerobic cultivation and slide culture methods were performed to support the *in vivo* findings described above, but it has been shown that these *in vitro* culture methods are inadequate for the resolution of the problem of the host-parasite relationship.

Experiments using the chamber technique showed that cortisone administration to rats has no marked influence on the effect of body fluid on the growth of virulent tubercle bacilli *in vivo*, especially in the enhancement of multiplication. This discrepancy of results between rats and rabbits might be questioned in the future.

REFERENCES

1) S. Tsuji et al. : Rec. Adv. in Res. Tuberc., **8**, 215, 1954.
2) S. Tsuji et al. : Rec. Med., **11**, 210, 1956 (in Japanese).
3) S. Tsuji et al. : Rec. Med., **11**, 134, 1956 (in Japanese).
4) S. Tsuji et al. : Am. Rev. Tuberc., **72**, 393, 1955.
5) K. Ito : Rep. Tub. Res. Instit. Kyoto Univ., **7**, 35, 1958 (in Japanese).
6) H. Yamamoto : Rep. Tub. Res. Instit. Kyoto Univ., **1**, 42, 1954 (in Japanese).
7) S. Tsuji et al. : Jap. Joun. Tub., **2**, 238, 1954.
8) T. Tao : Jap. Jour. Tub., **5**, 44, 1957.
9) H. Yamamoto : Act. Tub. Japan., **2**, 78, 1952.
10) S. Tsuji et al. : Rec. Med., **12**, 177, 1957 (in Japanese).
11) S. Tsuji et al. : Am. Rev. Tuberc., **77**, 529, 1958.
12) S. Tsuji et al. : Am. Rev. Tuberc., **77**, 524, 1958.

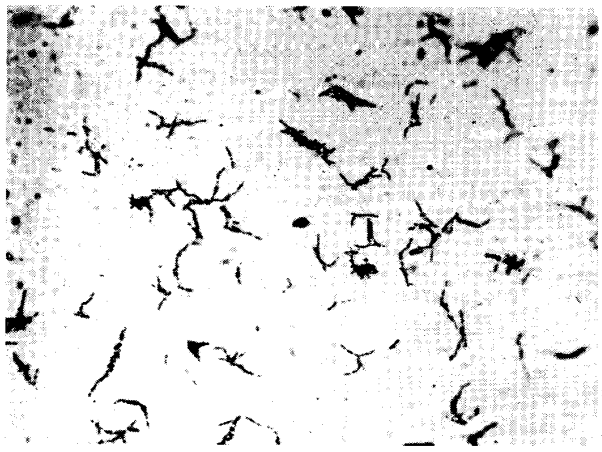

Fig. 1. Proliferation of H37Rv strain in the O-Chamber after one month (Rabbit). (900×)

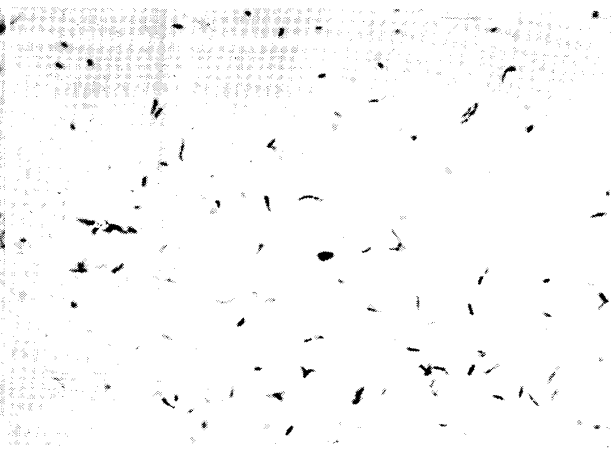

Fig. 2. Proliferation of H37Rv strain in the O-Chamber after one month (Rat). (900×)

Fig. 3. Proliferation of avian type (Cho-kyo) strain in the O-Chamber after three weeks (Rat). (900×)

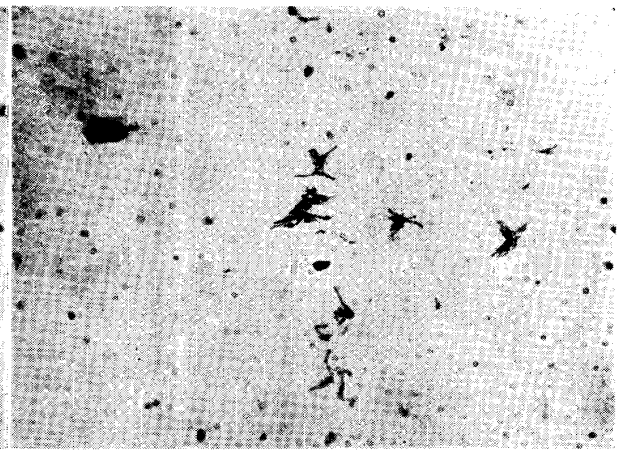

Fig. 4. Proliferation of BCG strain in the O-Chamber after one month (Rat). (900×)

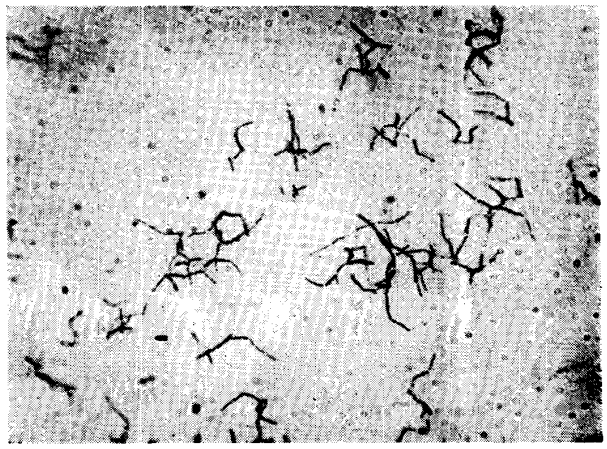

Fig. 5. Proliferation of Mycobacterium 607 in the K-Chamber after three weeks (Rat). (900×)