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Studies on prevention of infection (I) Antigenic capacity of enzymati-cally active fractions isolated from tubercle bacilli against tuberculosis of mouse

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Abstract

After grinding the tubercle bacilli cells, both human virulent strain, H37Rv, and avirulent strain, H37Ra, cultured in 5auton's medium, and obtaining three fractions of R1, S1 and R2 (R1, the first sediment; S1, the second supernatant; and R2, the second sediment) by the ultracentrifugation, the authors studied the enzymatic activities and the antigenic capacity against infection of these fractions; and obtained the following results: 1) Although the R1-fraction confers the defensive forte to mice in some degree, because of the presence of living bacilli in the fraction, it is difficult to decide definitely whether the defensive force owes its capability to this fraction or to living bacilli at the present stage of our experiment. 2) The S1-fraction possesses enzymatic activity on various substrates, but it does not confer animal any defensive force against infection. 3) The R2-fraction specifically oxidizes lactate and succinate" and it can markedly impart animal the defensive ability against infection.

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STUDIES ON PREVENTION OF INFECTION (I)

ANTIGENIC CAPACITY OF ENZYMATICALLY ACTIVE FRACTIONS ISOLATED FROM TUBERCULE BACILLI AGAINST TUBERCULOSIS OF MOUSE.

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The vaccination by BCG has amply verified general belief that the tuberculosis infection confers a definite immunity. DUBOS and his colleagues[1] state that essentially the antigenic capacity of tubercle bacilli depends upon the growth of the bacteria in the body of animal ; namely such a capacity is the result of a secondary increase of antigen in a body, and the strain like H37 Ra, which posseses no abilty of multiplication in animal body, is inferior to BCG[2] on the other hand, YAMAGUCHI[3] contends that the antigenic capacity of H37Ra is somewhat superior to that of BCG ; While CALMETTE and USTEVEDT state that the immunity can be maintained only so long as there exist live bacilli within an infected body.

However, the killed cells[4,5] of virulent bacillus by means of heating or action of phenol are also recognized to possess antigenic capacity, and in addition, substances[6] extracted from cells of bacilli by organic solvent are likewise supposed to possess antigenic capacity.

As far as could be deduced from these reports, it is extremely difficult to conclude at once that the tuberculosis infection can confer a definite immunity.

Recently YOUMANS et al[7] showed in their experiments with mice that the enzymatically active granules isolated from ground H37Ra by ultracentrifugation possess a strong antigenic capacity against the infection. YAMAMURA and his co-workers[8,9] state that granules isolated from the cells of avian type bacillus by ultracentrifugation can oxidize malate specifically and maintain the electron transfer system, and that these properties of granules resemble closely those of mitochondria in the animal cells.

At about the same time but independent of YOUMANS et al., the

authors commenced the study of immunization with the use of bacterial micro-particles possessing the enzymatic acitivity.

MATERIALS AND METHODS

Bacilli examined : The human virulent strain, H37Rv, and the avirulent strain, H37Ra, both strains are obtained from laboratory stock being kept through successive transfer once a month in Ogawa's neutral egg medium. For the ultracentrifugal preparation, the pellicle of bacteria cultured in Sauton's media for 16 days is used.

Fractionation by ultracentrifuge : The method is briefly shown in Table 1. Namely, the cells of bacteria are washed repeatedly, and about 13g wet cells obtained by collecting the washed bacteria on a sterilized filter paper is ground with quartz sand one hour at 0°C by means of mortar. To this ground thick paste about 50—60 ml of 0.02 mol phosphate buffer (pH 7.0) is added and after stirring well, the mixture is centrifuged 30 minutes at 4000 rpm. The supernatant so obstained is further centrifuged 40 minutes at 15000 rpm and the sediment (to be abbreviated as R_1 hereafter) is made into a suspension by adding 13 ml of 0.02 mol phosphate buffer (pH 7.0). This supernatant is again centrifuged one hour at 40000 rpm, and fractionated into the sediment R_2 and the supernatant S_1. R_2 is made into the suspension in exactly the same manner as the suspension on R_1. S_1 is used for experiment after removing the creamy fat substance floating on the surfce. All these procedures are undertaken at a low temperature.

Table 1.

Preparation of Particulate and Soluble Fraction

13 g (wet cell) + glass powder

grinding

1 hour at 0°C

thick paste + 0.02 M phosphate buffer pH 7.0

centrifuged at 4000 rpm for 30 min at 0°C

precipitate (discarded)

crude supernatant

centrifuged at 15000 rpm for 40 min at 0°C

precipitate (R_1)

supernatant

centrifuged at 40000 rpm for 1 hour at 0°C

precipitate (R_2) particulate

clear supernatant (S_1) soluble fraction

Experimental method: The oxygen uptakes at the time when various substrates are added are measured on the fraction S_1 and R_2 by conventional Warburg's techniques; and in the case of the antigenic capacity test by mouse the fractions, R_1, S_1 and R_2 are used.

RESULTS

A. *Enzymological properties of S_1 and S_2 fractions:*

As far the substrates the substances involved in the TCA cycle, such as pyruvate, lactate, acetate, citrate, succinate, fumarate, malate, etc, are mainly used. The content of each vessel of Warburg's manometer is 3 ml (the fraction 2 ml, substrate 0.3 ml, phosphate buffer 0.7 ml), and final concentration of substrate is made to 0.1 mol.

Figs. 1 and 2 show the amounts of the oxygen uptake by S_1 of H37Rv and of H37Ra respectively.

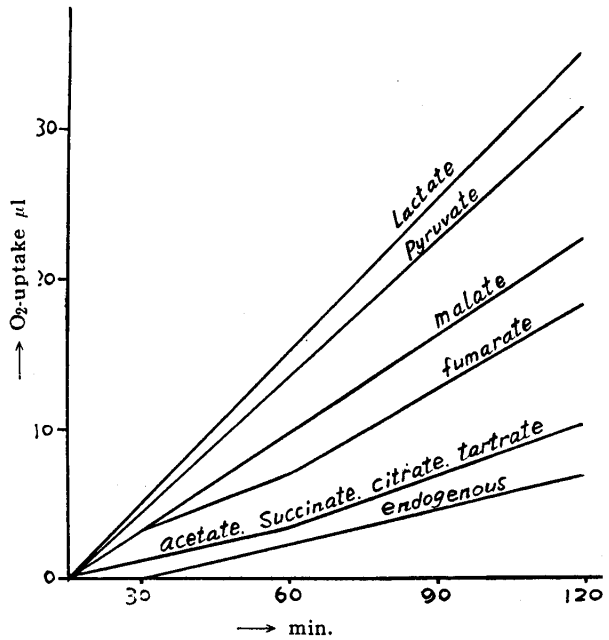

Fig. 1. The O_2-Uptake of Ultracentrifuged Supernatant from H37Rv

As shown in figures, both the S_1 fractions from H37Rv and H37Ra can oxidize well lactate, malate and fumarate: especially it is striking in the case where the substrate is lactate or pyruvate. In contrast to these, the oxidation of succinate, acetate and citrate is extremely low,

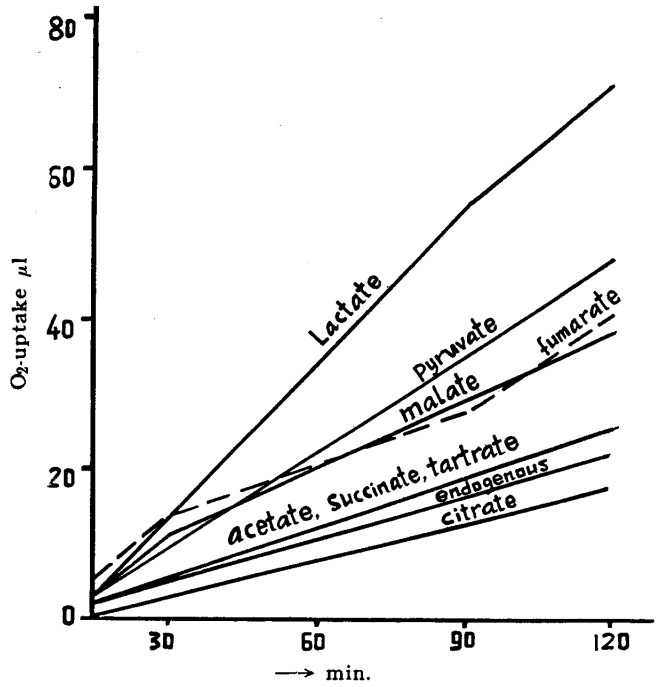

Fig. 2. The Oxygen Uptake of Ultracentrifuged Supernatant of H37Ra

showing hardly any difference from that of endogenous respiration. On the whole S_1 fraction of H37Ra showed a high oxygen uptake than that of H37Rv, and this seems to be due to the high endogenous respiration itself in the former.

The enzymatic activity of R_2 from H37Rv and H37Ra is shown in Fig. 3.

It is noticed that both S_1 oxidize substrates to greater extent and also show endogenous respiration to a certain degree; on the contrary, R_2, showing no endogenous respiration at all, oxidize specifically only lactate and succinate and cannot oxidize any of other substrates. From these resuls it seems that the oxidative enzyme system of lactate is localized in both S_1 and R_2, While that of succinate is localized in R_2.

B. *Antigenic capacity of R_1, S_1 and R_2 fractions from H37Rv*:

Normal mice weighing around 13—15 g were selected as the test animals, and these were divided into four groups: one injected with R_1, S_1 and R_2, and the control group, each group consistiong of 8 animals respectively. One half milliliter of each fraction was injected intraperitoneally, and a month later 0.5 mg of the virulent strain, H37Rv which

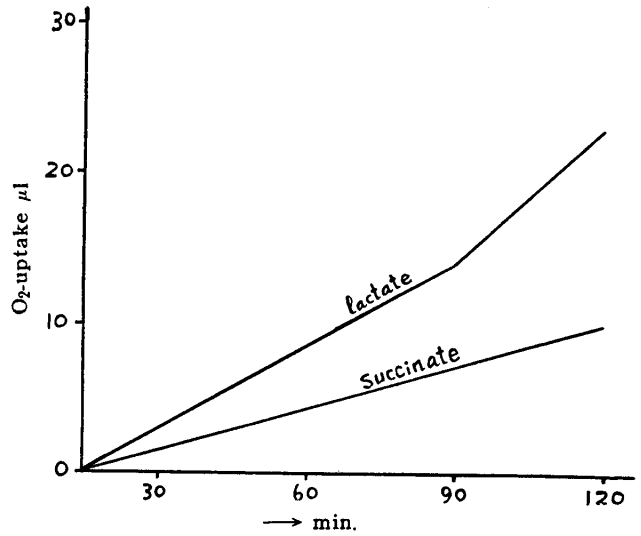

Fig. 3. Oxygen Uptake of Particulate Fraction of H37Rv

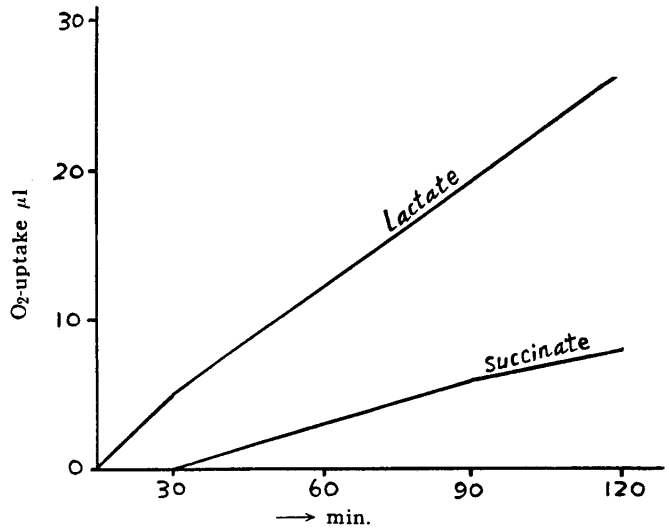

Fig. 4. Oxygen Uptake of Particulate Fraction of H37Ra

had been cultured for 18 days in Sauton's medium was inoculated intravenously.

Needless to say the control was not treated previously at all. Macroscopic observations of pathological changes in the viscera were carried out on the fortieth day, while those dead ones immediately after their

Table 2. Influences of the Fractions obtained by High Speed Centrifugation of H37Rv upon the Infectious Death of Mouse

Frations	Groups of the mice treated with different fractions a month after intraperitoneal treatment with 0.5 ml of each fraction, attacked (iv) with 0.5 mg H37Hv								Each fraction	
									culture	staining
15000 rpm R_1	22 ▨ ##	22 ▨ ##	25 ▨ ##	28 ▨ ##	○ ##	○ ##	○ +	○ +	##	+
40000 rpm R_2	35 ▨ ##	○ +	○ ±	○ ±	○ −	○ −	○ −	○ −	−	−
40000 rpm S_1	20 ▨ ##	21 ▨ ##	21 ▨ ##	23 ▨ ##	25 ▨ ##	25 ▨ ##	27 ▨ ##	○ ##	−	−
control	20 ○ ##	20 ○ ##	20 ○ ##	21 ○ ##	22 ○ ##	23 ○ ##	23 ○ ##	25 ○ ##		

Legends: ▨···mouse died by infection
##～−···degrees of macroscopic pathological changes in viscera
numerals···duration of survival

death. The results are shown in Table 2.

In the R_1-injected group one half of animals died within 22—28 days and irrespective of the dead or the survivings they all revealed various degrees of tuberculous changes. In the R_2-injected group, with the single exception that died on the 35th day, all survived the inoculation, and moreover, pathological changes in those surviving cases were extremely slight and macroscopically the majority of them revealed no changes. In contrast to this in the S_1-treated group, with only one survival, all others died within 20—27 days, and they showed extremely marked pathological changes. Naturally numbers of control group all died within 20—25 days after the inoculation, and they revealed pathological changes not greatly differing from those of the S_1-treated group. In these group the defensive force against pathogene was most striking in those treated by R_2, they were followed by those treated by R_1, but S_1 did not show protective effect at all.

Of these results the effect of R_1 is quite dubious inasmuch as this fraction demonstrated living bacillus both in staining and culture examinations, there is a sufficient reason to believe that live bacilli have induced immunity. Therefore, R_1 is the fraction composed of living cells and cell wall. And also, from the view of present knowledge there is a great doubt as to believe that the cell wall itself might possess the ability

to induce antibody.

Having observed that the R_2-fraction of H37Rv imparted a greater protective power against the infection, the authors, as the second step in the present study, investigated whether the R_2-fraction of H37Ra possessed the capacity in the same degree as the former and also made a comparison with R_2-fraction suspended in 0.02 mol phosphate buffer (pH 7.0) as above and the R_2-fraction suspended in liquid paraffin in place of the buffer sulution.

Similarly as in the previous experiment 0.5 ml of R_2 suspension of both strain was injected intraperitoneally into mice; and a month after the injection, these animals were inoculated with 0.5 mg of H37Rv intravenously.

At first just prior to the inoculation for the determination of whether or not the inoculation of such R_2-fractions would induce any pathological changes of tuberculosis in the viscera, two mice from each group were killed and their pathological changes were studied microscopically. The results are shown in Table 3.

Table 3. Pathological Findings one Month after R_2- Treatment (immediately before the attack)

group	No.	degree of inflammation			remark
		organs			
		liver	lung	spleen	
untreated control	1	−	±?	+?	numerous Staphylocicci in the lung, spleen is slighly infected
	2	−	−	−	
H37Rv R_2-buffer	1	−	−	−?	a typical giant cells in the spleen
	2	−	−	−	
H37Rv R_2-Paraffin	1	−?	−	−	a slight congestion of the liver
	2	−	−	−	
H37Ra R_2-buffer	1	−	−	−	
	2	−	−	−	
H37Ra R_2-Paraffin	1	−	−	−	
	2	−	−	−	

R_2-buffer···R_2 is mixed with 0.02 mol phosphate buffer (pH 7.0)
R_2-paraffin···R_2 is mixed with liquid paraffin

As shown in Table 3, the injection of R_2-fractions induced no pathological changes at all in the viscera, only in the spleen of the animal No. 1 of the group injected buffer suspension of R_2 from H37Rv revealed the

proliferation of unspecific giant cells, and the liver of the animal No. 1 of the group injected paraffin suspension of R_2 from H37Rv showed slight congestion ; but these can not be construed as important pathological changes. In the control, one mouse (No. 1) revealed a large number of cocci in the lungs along with a slight infection of the spleen, but these pathological changes, of course, occurring in the control group, clearly are not associated in any way with tubercle bacilli. From these facts it is presumably confirmed that the injection of R_2-fractions has not evoked any pathological reactions to the mouse viscera (lungs, liver, spleen).

The findings 10 days after the inoculation are shown in Table 4. In this case two animals were likewise selected from each group for autopsy and for the preliminary examination.

Table 4. Findings of Each Group 10 Days after the Attack

group treated	No.	inflammations								
		prolieferative			exudative			caseous		
		organs								
		liver	lung	spleen	liver	lung	spleen	liver	lung	spleen
untreated	1	±	±	±	−	−	−	−	−	−
control	2	−	±	±	−	−	−	−	−	−
H37Rv	1	±	±	−	−	−	−	−	−	−
R_2-buffer	2	−	±	−	−	−	−	−	−	−
H37Rv	1	±	±	−	−	−	−	−	−	−
R_2-paraffin	2	±	−	−	−	−	−	−	−	−
H37Ra	1	−	−	−	−	−	−	−	−	−
R_2-buffer	2	−	±	−	−	−	−	−	−	−
H37Ra	1	±	±	±	−	−	−	−	−	−
R_2-paraffin	2	±	−	±	−	−	−	−	−	−

Macroscopically only swelling of the spleen can be recognized in each group, and no other pathological changes can be observed.

In the microscopic observations, however, pathological changes can be seen in each group, but these changes hardly differ from those observed in the control group. Furthermore, although there ocurred pathological changes, these can not be recognized as tubercle formation specific to tuberculosis, and only they are limited to the infiltration of such cells as epitheroid cells and lymphocytes, and they are in reality only proliferative inflammation.

In other words, although 10 days after the inoculation some patho-

logical reactions *in vivo* can be recognized, no specific changes to tuberculosis can yet be seen. Table 5 shows the findings on the two mice each from respective groups killed 20 days after the attack.

Table 5. Findings of Each Group 20 Days after the Attack

group	No.	inflammations								
		proliferative			exudative			caseous		
		organs								
		liver	lung	spleen	liver	lung	spleen	liver	lung	spleen
untreated control	1	++	+	++	+	++	±	−	−	+
	2	+	+	++	±	+	+	−	−	+
H37Rv R_2-buffer	1	+	±	±	−	−	−	−	−	−
	2	+	±	−	−	−	−	−	−	−
H37Rv R_2-paraffin	1	+	±	+	−	−	−	−	−	−
	2	+	+	±	−	−	−	−	−	−
H37Ra R_2-buffer	1	±	+	±	−	−	−	−	−	−
	2	±	+	±	−	−	−	−	−	−
H37Ra R_2-paraffin	1	+	±	−	−	−	−	−	−	−
	2	+	±	−	−	−	−	−	−	−

Although the swelling of the spleen can be recognized in every group as in the previous experiment, the control group, in addition, give rise to the formation of fine tubercles in the liver.

In microscopic findings of the R_2-injected group a few fine-tubercle formations can be recognized, particularly in the liver and lungs, though in an extremely slight degree, but no exudative and caseous foci can be seen at all.

On the other hand, in the control group the formations of tubercle are marked in the lungs, liver, and especially so in the spleen ; and also exudative lesion of various degrees can be observed in the liver, lungs and in the spleen which reveals caseous lesion as well.

Namely, on the 20th day after the inoculation pathological changes specific to tuberculosis appear in every group. However, in the R_2-injected groups only a slight proliferative change can be observed, but in the control the exudative and caseous changes have occurred.

On the 33rd day after the inoculation all the mice surviving in the R_2-injected groups were killed and examined. These results are shown in Table 6.

In Table 6 the control group is not listed for they all died within 20

Table 6. Findings of Each Group 33 Days after the Attack

group	No.	inflammations								
		proliferative			exudative			caseous		
		organs								
		liver	lung	spleen	liver	lung	spleen	liver	lung	spleen
H37Rv	1	+	+	±	−	−	−	−	−	−
R_2-buffer	2	+	+	−	−	−	−	−	−	−
H37Rv	1	+	+	+	−	−	−	−	−	−
R_2-paraffin	2	+	±	+	−	−	−	−	−	−
	3	+	+	−	−	−	−	−	−	−
H37Ra	1	±	+	±	−	−	−	−	−	−
R_2-buffer	2	+	+	+	−	−	−	−	−	−
	3	±	+	+	−	−	−	−	−	−
H37Ra	1	+	+	+	−	−	−	−	−	−
R_2-paraffin	2	±	+	+	−	−	−	−	−	−

—29 days after the inoculation, but the results observed of these animals are summarily shown in Table 7.

As shown in Table 6, even on the 33rd day after the inoculation the pathological change in the R_2-injected groups is slight, all being proliferative ; and microscopically they showed only fine-tubercle formations or cell infiltrations in the lungs, liver and spleen, but they reveal neither exudative nor caseous changes. It gave an appearance that in all likelihood these proliferatiferative changes would shortly disappear.

Table 7 shows the results of the mice in the control group, died of infection within 21—29 days after the inoculation, and those of off-springs of the control, that were born just before the inoculation. These young mice likewise died within 20—29 days after the inoculation on the mother mouse (these off-springs numbered eight in all at the delivery, but the five that died within 2—3 days are excluded).

As shown in Table 7, all the proliferative changes is not so marked both in the control group and in the young mice born of the control; but their exudative and caseous inflammatory changes are exceedingly striking. The degree of the change is especially high in the two mice (No. 7 and No. 8).

Although not mentioned in Table 7, it is interesting to note that despite the fact that young mice born of mother mice of both the H37Ra-treated and the H37Rv-treated groups just before the inoculation, all survived, while the young mice born of the control group, evoking a high

Table 7. Findings of the Mice that died in the Control

group	No.	inflammations								
		proliferative			exudative			caseous		
		organs								
		liver	lung	spleen	liver	lung	spleen	liver	lung	spleen
control (untreated)	1	±	±	−	##	##	##	##	##	##
	2	+	+	±	#	##	##	+	##	##
	3	+	+	±	±	+	#	−	−	#
control (young mice born just before the attack)	1′	−	+	#	##	#	+	−	−	#
	2′	±	−	−	#	##	##	#	##	##
	3′	+	±	+	±	##	#	−	##	−

degree of tuberculous inflammation, all died. The authors have not yet studied the reason of a higher sensitivity of the off-springs born of the control compared with that born of R_2-injected groups, that survived.

DISCUSSION AND CONCLUSION

The antigen-antibody reactions that can be determined *in vitro* have made a great advance, but these reactions are still inadequate to explain sufficiently the defensive ability against infection. The fact that the antigen existing in serum does not directly contribute much towards the defense against infection has been reported by many workers such as WHITE[10,11] AVERY and MORGAN[12] LANCEFIELD et al[13]. However, as has been pointed out by DUBOS[14] there is at the present moment no theoretical explanation which may be settled definitely what structural components of bacterial cells are involved in the protective reaction against infection. However, it is possible to assume that the specific component or components in bacterial cells play a dominant role in such protective reation.

As for the research concerning the immunity of tuberculosis, there are many reports on BCG which is most widely studied, and the immunity induced by attenuated living cell, virulent killed cell, or cellular components of bacilli, recently the immunization by killed bacilli being in use with adjuvant is being studied widely[15~21]. Furthermore, YOUMANS et al[7] state that enzyme active micro-components isolated from ground tubercle bacilli by ultracentrifugation are deeply involved in the protection against infection ; and YAMAMURA[9] has succeeded in excavation with the substances corresponding to these.

Believing that the antigen defending infection is not the identical substance as the antigen existing in serum and that what makes it possible

to produce this antigen is specific enzyme active, fine structured component or components in the bacterial cells, the authors commenced the investigation about the same time as did YOUMANS and his co-workers. For the determination of the capacity there are methods such as the death by infection, the tissue culture of organ for quantitative determination, and pathologic findings of viscera on which emphasis may be placed, but in the present report the emphasis is placed on the death by infection and pathological findings of viscera and the tissue culture method of quantitative determination is left for future studies.

Although the R_1-buffer fraction of the human virulent strain H37Rv, is not shown in Table, the enzymologic properties of this fraction closely resemble those of the S_1-buffer fraction, though their activity is extremely low. Admitting that this fraction confers in some measure the protection against infection, but as the examination by staining and the culture of this fraction demonstrates living bacilli, it is difficult to establish whether the antigenic capacity is dependent on living cells or on the fractions (in all likelihood the cell wall).

The S_1-buffer fraction of both the virulent strain, H37Rv, and avirulent strain, H37Ra, oxidizes lactate, pyruvate, malate and fumarate but it hardly or does not at all oxidize succinate, acetate and citrate. Moreover, the R_2-buffer fraction of the two strains specifically oxidizes lactate and succinate, but it does not at all oxidize any other substrates. Moreover, the S_1-buffer fraction could not stimulate the production of defensive antigen; and the group of mice injected with this fraction revealed the marked pathological change characteristic to tuberculosis similarly as the control group, they died of infection within 20 to 30 days after the inoculation. On the other hand, the R_2-buffer treated group, when compared with other groups, showed a marked protective ability against infection; and there was only one case that died of infection within 40 days after the inoculation. Furthermore, in autopsy findings of those surviving mice, macroscopically pathological changes could hardly be recognized or none at all.

Therefore, on the assumption that the R_2-fraction must be the main site of the protective antigen and in order to pursue it still further in detail, the R_2-fractions of H37Rv strain and H37Ra strain are both divided into two, namely, the one suspended in 0.02 mol phosphate buffer (pH 7.0) and the other suspended in liquid paraffin; and injected both suspensions of R_2 in two groups of mice separately, then after each groups of mice were inoculated the virulent strain of tubercle bacillus as before, these test animals were killed for autopsy 10, 20 and 33 days after the

inoculation and the pathological findings of their viscera were studied. In either of the R_2-injected groups even on the 33rd day showed only slight,proliferative changes, while the control group, already on the 20th day demonstrated exudative and caseous changes. Moreover, those dead mice in the control group showed marked exudative and caseous changes.

From these results it has been clarified that the R_2-fraction of H37Ra strain possesses an antigenic capacity not in any degree inferior to that of H37Rv, and therefore, the R_1-fraction needs not necessary to be added liquid paraffin as an adjuvant. At present the authors are conducting a series of experiments to find out more precisely the chemical nature of the R_2-fraction or the location of the fraction in the bacterial cell.

SUMMARY

After grinding the tubercle bacilli cells, both human virulent strain, H37Rv, and avirulent strain, H37Ra, cultured in Sauton's medium, and obtaining three fractions of R_1, S_1 and R_2 (R_1, the first sediment; S_1, the second supernatant; and R_2, the second sediment) by the ultracentrifugation, the authors studied the enzymatic activities and the antigenic capacity against infection of these fractions; and obtained the following results:

1) Although the R_1-fraction confers the defensive force to mice in some degree, because of the presence of living bacilli in the fraction, it is difficult to decide definitely whether the defensive force owes its capability to this fraction or to living bacilli at the present stage of our experiment.

2) The S_1-fraction possesses enzymatic activity on various substrates, but it does not confer animal any defensive force against infection.

3) The R_2-fraction specifically oxidizes lactate and succinate, and it can markedly impart animal the defensive ability against infection.

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