大韓微生物學會誌 第5卷 第1號 1970
J. Kor. Soc. Microbiol., Vol. 5, No. 1, 1970

Effect of Immune Serum on the survival and Respiration of Brucella

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—국문초록—

Brucella 면역혈청이 균생존과 호흡에 미치는 영향

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정상혈청과 면역혈청의 존재하에 있어서 균의 생존율이 호흡율과 어떠한 관계가 있는가 Brucella 균을 사용하여 실험해보았다. 혈청의 원액에 균을 37°C 3시간 방치했다가 흔히 쓰는 평판주입법에 의하여 생존균수를 측정했고, 균호흡은 Warburg 장치를 사용하여 측정했다. 그 결과를 요약하면 다음과 같다.

혈청의 살균작용과 균호흡억제작용 간에는 극히 대조적인 현상을 엿볼 수 있었는데, 그것은 균호흡이 억제되면 될수록 생존균수는 그만큼 증가되었다는 사실이다.

Brucella 면역혈청의 살균계수가 1.4~1.6이었는데 반해 정상혈청은 2.7나 되었으며, 그 차는 무려 $\log_{10}$ 일단위 이상이나 되었다. 그러나 면역혈청내에서의 균호흡율은정상혈청중에서의 호흡율의 반도 안 되었다. 면역체가 보체와 더불어 이러한 호흡억제 작용을 나타낸 것 같으며 이렇게 호흡이 억제된 상태에 있는 균은 혈청의 살균인자에 보다 무감각한 것 같다. 즉 in vitro에서 Brucella 면역체는 어느 일정량 이상 존재하면 균호흡을 억제하고 동시에 혈청의 살균인자로 부터 균을 보호해 준 역할을 한다고 볼 수 있다.

ABSTRACT

The survival rate of Brucella in the immune and normal serum was compared with the respiration rate of cells in the presence of the serum. The Brucella were suspended in the serum of original concentration and incubated at 37 C for 3 hr. Then, the cells that survived were counted by the routine pour plate method. As to the measurement of bacterial oxygen uptake the standard manometrie techniques were applied.

Contrasting results were observed between the serum bactericidal activity and the respiration of Brucella. The bactericidal index of Brucella immune serum was 1.4 to 1.6 while 2.7 at normal serum, the difference being more than one $\log_{10}$ unit, whereas the oxygen uptake

rate of Brucella in their homologous immune serum was less than a half of the rate in normal serum. Immune bodies exerted the inhibitory effect together with complement on the oxidative metabolism of Brucella. Addition of various substrates, such as D-alanine, L-glutamic acid, and glucose, into the Warburg flask did not bring about any noticeable change in the effect of immune and normal serum on the bacterial metabolism.

INTRODUCTION

It is no longer questioned in most of the serum sursceptible Gram negative organisms that the bactericidal activity of immune serum is significantly lowered as compared with that of normal serum when the serum tested is not diluted and no excessive amout of exogenus complement is supplied. This reduced bactericidal activity is particularly distinguished in brucellosis (4, 5, 11). Utilizing such characteristics of Brucella and their immune serum, Minoprio (11) has developed even a laboratory technique to differentiate the brucellosis from other chronic diseases. The mechanism involved in this prozone inhibition phenomenon is, however, so complicated that it is still waiting to be solved definitely. The author, pursuing an answer to this problem, has tried to compare the respiration rate of Brucella in the presence of immune and normal serum as one of the steps to find a clue to this subject.

In previous reports (1, 2), the effect of gamma irradiation on the respiration of various organisms has been investgated with the result that cells respired appreciably even after the lethal doses of gamma rays were given. Youmans (19), working on the effect of on the respiration acitvity of M. tuberculosis. has reported that the difference of serum, i. e., immune or normal, did not have any effect on the oxidative metabolism while lung cells from the vaccinated guinea pigs affected the activity considerably.

The present experiment has. however, produced a seemingly discordant finding that immune bodies exerted a great inhibitory effect on the respiration of Brucella in the presence of serum.

MATERIALS AND METHODS

Organism. Brucella abortus strain 19 and Brucella melitensis were used in this experiment. The cells were cultured on Brucella Agar (Difco) slants for 48 hr at 37 C. The cultures were harvested and washed twice in physiological saline, and then suspended in saline or Sorensen's phosphate buffer solution (pH7. 0).

Serum. Rabbits were injected through the ear vein with heat-killed (60 C 30 min) suspensions of the organism. The concentration of the suspension was approximately 1×10^{10} per ml. The antigen was injected four times at intervals of three days; initial dosage being 0. 5 ml, second 1 ml, third 2 ml, and fourth 4 ml. Immunized rabbits were bled by heart puncture one week after the last injection. Serum was seperated after leaving the blood clotted at room temperature for 2 hr and then at 0 C overnight.

Manometric technique. The respiratory rate of the cell was measured by the standard method of Umbreit (18), following the modified procedure of Meyer (10) as to its specialized detaills. The temperature of the water bath was 37 C. The temperature equilibrating time was 15 min before and after tipping in the substrate, respectively. Each Warburg flask contained 1. 4ml of Sorensen's buffer solution containing the serum at the ratio of 1:1 and 1 ml of cell suspension in the main chamber, 0. 5ml of substrate solution in the side arm, and 0. 1ml of 20% KOH solution in the center well. Mannometric readings were taken at every 30 min for 3 hr. This was repeated on several harvestings of cells and sera, and the results

shown in tables are the average of the duplicate determinations.

The substrates were prepared by dissolving the compounds in Sorensen's buffer solution and adjusting the pH, when necessary, by the addition of NaOH. One per cent solution of D-alanine, L-glutamic acid, and glucose were used as the substrates (1, 10).

All oxygen uptake rates were expressed in microliters of oxygen consumed per hour per 1mg nitrogen of cells. It is shown in tables as QO_2 per mg N.

Serum bactericidal activity. Saline suspensions of the fresh Brucella abortus in a concentration of 2×10^8 per ml were employed in the bactericidal reaction test. 1ml of the cell suspension was put into the test tubes containing an equal amount of undiluted serum. After enough stirring, the tubes were incubated at 37 C for 3 hr. The number of cells that survived this treatment were counted by the routine pour plate method. The bactericidal index of serum was expressed by

$$\log \frac{\text{initial viable count}}{\text{final viable count}}$$

Table 1. Effect of immune bodies on the serum bactericidal activity to Brucella

Serum	Agglutination titer (reciprocal)	Bactericidal index
Normal	<20	2.7
Anti-Br. abortus	1280	1.6
Anti-Br. melitensis	2560	1.5

Table 2. Effect of immune bodies on the respiration of Brucella

Serum	Agglutination titer (reciprocal)	QO_2 per mg N	
		Br. abortus	Br. melitensis
None	—	13	15
Normal	<20	295	315
Anti-Br. abortus	1280	115	145
Anti-Br. melitensis	2560	132	93

RESULTS

Reduced bactericidal activity of immune serum. The bactericidal activities of rabbit's normal and immune serum without an exogenous addition of complement were compared to each other (Table1). The bactericidal index of the immune serum was appreciably lower than that of the normal one. Between the immune sera no particular difference was noticed, so far as the agglutination titer was within a certain limited range. There was, however, a tendency that the agglutination titer of serum gives a significant inverse influence on the bactericidal activity, i.e., the lower the titer, the higher the serum bactericidal activity.

Respiration of cells in serum. Generally the respiratory rate of cells in serum was unexpectedly high. The serum itself could rapidly be utilized for the oxidative metabolism of the cell. The amount of oxygen consumed by the cell in the presence of normal serum was, however, noticeably greater than that consumed in the presence of immune serum, regardless of the kind of organism and heterogeneity of the serum (Table2). These results would indicate that serum plays a role like a substrate in the bacterial respiration but some agents in immune serum seem to exert an inhibitive effect on it.

Table 3. Respiration of Brucella in the presence of heat-inactivated serum

Heat-inactivated serum	Agglutination titer (reciprocal)	QO_2 per mg N	
		Br. abortus	Br. melitensis
Normal	<20	357	397
Anti-Br. abortus	1280	315	348
Anti-Br. melitensis	2560	332	329

Effect of heat-inactivation of serum on the respiration. When the serum was inactivated by heating at 56 C for 30 min and submitted to this test, the differences in the bacterial respiration rate were not noticed between the normal and

immune serum nor between the immune sera, suggesting that the complement played a contributory role in inhibiting the rate in immune serum while in the normal one it was negligible (Table3). The agglutination titers of immune sera submitted to this test so far were high enough to render the cells agglutinated in the Warburg flask during the experimental proceedings. Therefore, the probable reduction of bacterial surface due to this agglutination reaction might be thought as a possible cause of the decrement in the respiration rate. But, as shown above, although the agglutination was not affected at all by the inactivation process, the respiration rate was greatly increased. This finding strongly suggests that the antibodies in cooperation with the complement would would work as inhibitory agents in the bacterial respiration.

Effect of immune body absorption. In order to prove the effect of immune bodies more directly, the antibody absorption reaction was carried out at 37 C for 3 hr and the bacterial respiration rate was measured in the presence of the absorbed sera. Then, the results were encocouragingly coincidental (Table4), showing that the absorption reaction brought about an appreciable inhancement in the oxidative metabolism. It is likely that the damage given to the complement by the absorption process may cause partially the increment in the respiration rate. But the control, in which fresh exogenous complement was added in the ratio of 1:1, did not bring about any particular differences. Once the antidodies were depleted, the complement became completely inert in having any influence on the respiration.

Table 4. Respiration of Brucella abortus in the presence of absorbed serum

Serum	Treatment of serum	Agglutination titer (reciprocal)	QO_2 per mg N
Normal	None	<20	295
	Absorbed	<20	344
	Complement added after absorbed	<20	339
Anti-Br. abortus	None	1280	115
	Absorbed	40	284
	Complement added after absorbed	20	298

Respiration under substrates and serum. In the tests described above, the side arm of the side arm of the Warburg flask did not contain any fluid since serum diluted in Sorensen's buffer solution was directly poured into the main chamber. But, this time the side arms were filled with 0.5ml of substrate solution. The addition of substrates to the serum did not amplify the differences between the immune and normal sera, only increasing the whole oxygen uptake rate proportionately, as shown in Table 5.

DISCUSSION

Many investigators (4, 5, 11-15) have reported that the bactericidal activity in immune sera

Table 5. Respiration of Br. abortus in the presence of substrate and serum

Serum	Substrate				
	D-alanine	L-glutamic acid	L-asparagine	Fructose	Glucose
None	105**	285	236	104	205
Normal	376	498	495	377	386
Anti-Br. aborts	198	317	310	201	208
Heated normal*	428	515	507	420	435
Heated anti-Br. abortus*	388	487	476	385	403

* 56C for 30min.

**QO_2 per mg N.

against Gram negative organisms is appreciably reduced, compared with that of normal serum, when the concentration of serum tested is within a range close to the original and no excessive exogenous complement is added to the system.

The goal of the present study was to find out what effect such an immune serum of lowered bactericidal activity would have on the respiratory metabolism of Brucella. The results, however, were seemingly contradictory, since a higher oxidative metabolic rate was observed in the serum which yielded lower survival rate of cells.

Youmans et al. (19) has reported data that serum from either immunized or non-immunized animals failed to inhibit the respiration of M. tuberculosis presumably because of the presence of too few inhibitng factor(s) or the presence of too many respiration stimulating materials while dialyzed lung homogenates from immunized guinea pigs showed an inhibitory action on the respiration of cells. Our interest was not in searching for the inhibitory factor(s) as contained in the tissue homogente which suppressed even the endogenous respiration of cells, but in comparing the respiration rate of cells in the presence of immune and normal serum, both of which would naturally stimulate to some degree the oxidative metabolism of cells since serum itself can serve as a kind of multiple substrate. The oxygen uptake rate of M. tuberculosis is extremely low compared with Brucella strains (1). And, it is a well-known fact that M. tuberculosis is generally very slow in any metabolic activities. The discordant findings, therefore, may ascribe to the differences in the nature of the test organisms as well as in the standpoint of the experiment.

The amount of oxygen consumed by the cells in the presence of serum obtained from the rabbit immunized with heat-killed Brucella was less than half the one consumed by the cells in the presence of normal rabbit serum. These results suggest that either less respiration stimulating factor(s) or more respiration inhibiting material(s) must be contained in the immune serum than in the normal one. The oxygen uptake rate of Brucella in the presence of only Sorensen's buffer solution was less than one tenth the rate under the serum or substrate. It is, therefore, more reasonable to assume that the serum in general played a role in the exogenous respiration of Brucella as a kind of complex substrate rather than serving as a stimulating factor for endogenous respiration. The invariable difference between the immune and normal serum would then be derived from the presence of some inhibiting factor(s) in the serum obtained from immunized animals, the antibodies. The antibody depleted immune serum did not show the inhibitory effect on the respiration as was observed in the non-absorbed immune serum (Tabl 4). This suffices to prove that the inhibitory effect originated from the presence of immune body.

Prozone inhibiton phenomenon occurs because of the presence of too many antibodies in the serum. When such a serum is diluted appropriately the bactericidal activity increases usually far beyond the level of normal serum. Muschel (12, 14) enumerated the amount of antibodies necessary for the strong bactericidal activity as less than one per cent of the amount to cover the whole surface of a cell. He also pointed out that the role of complement in serum bactericidal activity is indispensable. When sufficient complement is supplied, he asserted, even the concentrated immune serum would exhibit a greatly inhanced bactericidal activity.

Insufficient evidence exists as to the effect of these cotroversial in vitro reactions of immune serum on the immunological defense mechanism in vivo. Elberg (3) observed that persistent

inhibition of cellular degeneration induced by M. tuberculosis was noticed when immune cells were cultivated in immune serum, while cells in normal serum showed no significant resistance. Works by Jenkin et al. (7, 9) show that unless Gram negative bacteria have been opsonized they are not phagocytized by mouse macrophages, and in the case of Brucella such interaction of antibody was reflected in the increased survival of infected immune cells as compared with infected normals cells. In regard to this effect of immune serum on the intracellular killing of bacteria, much evidence has also been published(6, 8, 16, 17,). Thus, the decreased bactericidal activity of concentrated immune serum is yet to be investigated.

Old and metabolically dorment bacteria are usually less susceptible to harmful agents, such as irradiation and antibiotics. The results of the present experiment that Brucella respire less while surving longer in the presence of immune serum as compared with in the presence of normal serum may, therefore, contribute considerably in explaining the mechanism that Brucella is apt to undergo an exceptionally chronic course frequently.

ACKNOWLEDGMENTS

I acknowledge the excellent technical assstance of T. Y. Ha. I am also greatful to M. E. Meyer for her kindly gift of the Brucella strains.

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