EFFECT OF HOMOGENATES OF ORGANS FROM IMMUNIZED GUINEA PIGS ON THE RESPIRATION OF MYCOBACTERIUM TUBERCULOSIS

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An outstanding feature of the phenomenon of acquired immunity in tuberculosis is the lack of multiplication of virulent mycobacteria in the tissues of the immunized host. As the result, viable, virulent, tubercle bacilli may remain dormant in vivo for long periods of time.

The nature of the acquired specific mechanism responsible for this bacteriostasis in vivo has never been determined although there are reports which suggest that growth inhibitory substances may be present in the blood, body fluids, or tissues of immunized or infected animals (Pagel, 1935, 1940; Lurie, 1936, 1939; Myrvik and Weiser, 1951; Soltys, 1952, 1953; Tsuji, Ito, and Oshima, 1957). The role, if any, of most of these growth inhibitory substances in the production of the state of "physiological imprisonment" (McDermott, 1958) of virulent mycobacteria in the tissues of immunized animals has never been defined. Lysozyme, the only one which has been identified, does not increase the resistance of normal mice to tuberculous infection when administered in large quantities (Weiser et al., 1958).

Regardless of the role of growth inhibitory substances in immunity to tuberculosis, it is possible that certain cells of the immunized animals might acquire the ability to reduce the physiological activity of viable virulent mycobacteria to a level incompatible with reproduction, and thereby account for a significant portion of the acquired immunity. If such a mechanism is involved, it would be reasonable to expect that cells, or homogenates of organs obtained from immunized animals, might interfere with some essential metabolic function of virulent mycobacteria.

In support of this hypothesis the results to be presented will show that tissue homogenates prepared from the lungs of guinea pigs immunized with BCG vaccine have the capacity to inhibit the endogenous respiration of virulent tubercle bacilli.

METHODS

Guinea pigs weighing between 500 and 600 g were distributed randomly into cages and separated into three groups of equal size. The animals of the first group were not vaccinated and served as controls. The animals comprising the second group were vaccinated intraperitoneally with 5.0 mg of BCG vaccine and those of the third group with 1.0 mg of BCG vaccine.*

Unless otherwise indicated, five guinea pigs of each group were sacrificed by etherization every 2 weeks, and the lungs and spleens removed aseptically. At autopsy, blood was removed from the thoracic cavity by aspiration with a 10-ml syringe after the great vessels had been severed. The blood was allowed to clot and the serum collected aseptically and stored at 4 C. Each organ was washed in sterile 0.01 M phosphate buffer, pH 7.0, to remove excess blood, blotted semidry with sterile filter paper, and weighed. The lungs were placed in a small Waring Blender cup and 4.0 ml of the phosphate buffer solution were added for each gram of tissue. The tissues were homogenized for 30 sec at low speed and for 2 min at high speed in a 4 C cold room. Measurements revealed that at no time during the homogenization process did the temperature inside the cup exceed 40 C. The homogenates were placed in sterile pyrex tubes, covered, and stored at 4 C. The spleens were treated in a similar manner except, because of their small size, homogenization was accomplished by using a sterile Teflon grinder rotating at high speed.

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* Obtained from Dr. Sol Rosenthal, Tice Clinic, University of Illinois, Chicago, Illinois.
at 1,100 rpm in a matching tube for 5 min. After these procedures, and allowing the clumps of fibrous tissue to settle to the bottom of the tube, microscopic examination revealed no intact tissue cells.

Suspensions of washed cells of the virulent H37Rv strain of Mycobacterium tuberculosis were prepared in the manner described previously from cultures grown as surface pellicles on a modified Proskauer and Beck medium. The concentration of cells in the suspension was measured by centrifugation of a portion in a Hopkins vaccine centrifuge tube, following which the suspension was diluted to a concentration of 100 mg per ml with 0.01 M phosphate buffer, pH 7.0 (Youmans and Karlson, 1947).

A standardized Warburg procedure described earlier (Youmans, Millman, and Youmans, 1956) for measuring the oxygen uptake in air at 37.0 C of virulent tubercle bacilli was employed. Each Warburg vessel contained 0.5 ml of the cell suspension and 0.5 ml of 0.01 M phosphate buffer, 1.0 ml of tissue homogenate or serum in one side arm, and 0.2 ml of 10 per cent KOH in the center well. The endogenous respiration of the tubercle bacilli and of the tissue homogenates was determined by including Warburg vessels in which either tissue homogenate or the mycobacterial suspension was replaced with phosphate buffer. After a 15-min equilibration period, the contents of the side arms were tipped into the vessel and the equilibration continued for another 15 min before the manometers were adjusted and closed. Readings were made at 30-min intervals for 3 to 4 hr.

The homogenates were dialyzed by placing samples into separate cellophane sacs and dialyzing against three 1000-ml volumes of distilled water over a period of 18 hr. The adequacy of this period of dialysis was determined initially by testing homogenates dialyzed for both 18 and 36 hr. No differences could be detected between the two preparations.

RESULTS

It was found that dialysis removed some of the substrates from the lung and spleen homogenates which increased the respiration of the mycobacterial cells. Dialysis, however, did not affect the respiration inhibiting activity of homogenates prepared from the lungs of vaccinated guinea pigs. Therefore, only the results using dialyzed material have been given in table 1.

Homogenates prepared from the nonimmunized guinea pigs increased the respiration of the cells markedly, and, although there was some variation in individual results, the mean QO2 values of the homogenates from the 5 animals sacrificed at each time remained about the same throughout the 12-week period except for one high value obtained at the 6th week. Homogenates prepared from the lungs of guinea pigs immunized 2, 4, and 6 weeks previously with 5.0 mg BCG increased respiration as much as homogenates prepared from the lungs of control animals. However, lung homogenates prepared from animals 8 weeks after vaccination increased the respiration much less than the control homogenates. By the end of the 12th week these preparations actually inhibited the endogenous respiration of the mycobacterial cells. A similar pattern was obtained with homogenates prepared from the lungs of guinea pigs vaccinated with 1.0 mg of BCG. In view of the fairly wide variation noted between the effect of individual homogenates on the respiration of tubercle bacilli the data was subjected to an analysis of variance. It was found that statistically the mean QO2 values of the nonvaccinated animals did not differ significantly (P = >.01) during the 12-week period, whereas the mean QO2 values of both groups of vaccinated animals were significantly different (P = <.001).

Although the results are not shown in table 1, five animals vaccinated with 1.0 mg BCG were sacrificed at 23 weeks and 10 were sacrificed at 27 weeks. The lung homogenates from the former animals inhibited the endogenous respiration of the mycobacterial cells from 21.2 to 36.4 per cent. Eight out of 10 lung homogenates of the latter group inhibited the endogenous respiration from 32.1 to 57.1 per cent, one homogenate had no effect on the endogenous respiration, and one increased respiration.

The pattern of inhibition of the endogenous respiration of virulent tubercle bacilli noted with individual homogenates varied (figure 1). Inhibition could occur immediately and respiration then would proceed at a rate less than the endogenous during the entire experiment. At times respiration would proceed at the same rate as the endogenous for 1½ to 2 hr before decreasing. Finally, sometimes the respiration would be
### TABLE 1

*Effect of homogenates prepared from the lungs of vaccinated and nonvaccinated guinea pigs on the respiration of *Mycobacterium tuberculosis*

<table>
<thead>
<tr>
<th>Weeks after Vaccination</th>
<th>BCG Vaccinated</th>
<th>Nonvaccinated</th>
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<tr>
<td></td>
<td>5.0 mg</td>
<td>1.0 mg</td>
</tr>
<tr>
<td></td>
<td>No.</td>
<td>QO₂*</td>
</tr>
<tr>
<td>2</td>
<td></td>
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</tr>
<tr>
<td>1J</td>
<td>1.12</td>
<td>1K</td>
</tr>
<tr>
<td>2J</td>
<td>1.06</td>
<td>2K</td>
</tr>
<tr>
<td>3J</td>
<td>1.06</td>
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<td>4K</td>
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<tr>
<td>6J</td>
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<tr>
<td>7J</td>
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</tr>
<tr>
<td>8J</td>
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</tr>
<tr>
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</tr>
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</tr>
<tr>
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<td>0.72</td>
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</tr>
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<td>30K</td>
</tr>
<tr>
<td>Mean</td>
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<td>0.06</td>
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</table>

* Oxygen (μl) taken up per mg, wet weight, bacterial cells per hour corrected for the endogenous respiration.

† These nonvaccinated guinea pigs were killed at 9 weeks instead of 8.

The lung homogenates alone had QO₂ values between 0.04 and 0.08.

The QO₂ values of the endogenous respiration of the bacterial cells varied between 0.46 and 1.10; most of the values were around 0.60.
greater than the endogenous for 1½ to 2 hr, only to be less than the endogenous for the rest of the experiment. The QO2 values given in table 1 were calculated from the portion of the curve which showed inhibition of respiration. The delay of inhibition did not indicate a decrease in the amount of substrate, because, when these homogenates were diluted 2- to 8-fold they increased the respiration of the cells for the entire period of the experiment. When homogenates prepared
from the lungs of nonvaccinated guinea pigs were diluted, the amount of oxygen utilized decreased proportionately.

In figure 2 the effect of homogenates prepared from the lungs of the 5.0 mg vaccinated and of the nonvaccinated guinea pigs on the endogenous respiration of *M. tuberculosis* has been graphically represented. The slopes and Y intercepts of the lines shown on the graph were determined by the method of least squares using the results of the individual determinations made during the 12-week period. The increased capacity of homogenates obtained from the lungs of the guinea pigs immunized with 5.0 mg BCG to inhibit the respiration of virulent tubercle bacilli is indicated by the negative slope of the line in figure 2.

Dialyzed lung homogenates from the three groups of animals were retested after standing for 3 days at 4.0 C to determine whether these "steeped" homogenates would contain more of the inhibiting substance than was found in the 24-hr samples. The results indicated that standing did increase moderately the capacity of homogenates from vaccinated animals to inhibit respiration.

Experiments were conducted to determine the effect of the inhibitory lung homogenates on the oxidation of lactate and pyruvate (Youmans et al., 1956), and on the oxidation of the substrates present in homogenates of the lungs of nonvaccinated guinea pigs. The homogenate which inhibited the endogenous respirations of the mycobacterial cells also inhibited the oxidation of these substrates; thereby, providing direct evidence for the presence of a respiration-inhibiting substance(s).

The results obtained with spleen homogenates were somewhat more irregular, but, there was no convincing evidence that homogenates prepared from the spleens of vaccinated guinea pigs increased mycobacterial respiration to a lesser degree than homogenates prepared from the spleens of nonvaccinated guinea pigs.

Sera obtained from the vaccinated animals increased the respiration of the cells to the same degree as the sera obtained from the nonvaccinated animals.

**DISCUSSION**

The results presented in this paper indicate that following the immunization of guinea pigs with BCG vaccine the lungs of these animals gradually accumulate some substance(s) capable of markedly inhibiting the endogenous respiration of virulent tubercle bacilli. The detection of the substance(s) in the lungs of guinea pigs which inhibits respiration of tubercle bacilli is complicated by the release in the homogenization process of substances which increase the oxygen uptake. The ratio between the amount of respiration increasing and respiration inhibiting substance(s) will determine the rate of oxygen uptake. Only when the amount of inhibitor present is sufficient to completely counteract the respiration increasing, or stimulating, substances will an actual inhibition of respiration be observed. Until such a concentration is reached the presence of the inhibitor can only be inferred from the reduction in the respiration increasing effect of lung homogenates. To date, efforts to separate completely the inhibitor(s) from the respiration increasing substances have not been successful.

Caution should be exercised before too much significance is attached to these findings in relation to the mechanism of acquired immunity to tuberculosis. The capacity of the lungs to inhibit the respiration of tubercle bacilli may have developed only coincidentally with other changes more significantly related to increased resistance to infection. On the other hand, the demonstration of the presence of a respiration-inhibiting factor(s) in the lungs of immunized animals provides a plausible explanation for the state of dormancy of tubercle bacilli which so characteristically occurs in the tissues of immunized animals, especially when the complete dependence of tubercle bacilli on molecular oxygen for respiration is taken into consideration. A specifically acquired capacity of tissue to inhibit the respiration of virulent microorganisms would constitute a unique immune mechanism. However, a role for the respiration-inhibiting factor(s) in immunity to tuberculosis can be postulated only when it can be shown that increased resistance to tuberculous infection will follow the administration of this substance(s) to normal nonimmunized animals.

The failure to demonstrate any inhibition of respiration of *M. tuberculosis* by the homogenates of spleens or livers of immunized animals may be related to the greater susceptibility of these two organs in the guinea pig to tuberculous infection (Griffith, 1930). Therefore, the amount of respiratory-inhibitory substance(s) present in
these organs may have been too small to be
detected by the methods employed in the present
study. There is also the possibility that the
presence of much larger amounts of respiration-
inhibiting substances in these organs and their
release by the homogenization process may have
completely counteracted the action of the
respiration-inhibitory factor(s) present. The
development of procedures for the more complete
removal of oxidizable substrates from the
dialyzed homogenates of spleens and livers may
eventually reveal the presence of respiration-
inhibiting substances in these organs.

The failure of serum from immunized animals
to inhibit respiration also may have been due to
the presence of too little inhibiting factor(s) or to
the presence of too much respiration stimulating
material. Raffel (1933) also has reported that
serum from immunized animals or human beings
failed to inhibit the respiration of tubercle bacilli.

SUMMARY

Eight weeks or more after vaccination a non-
dialyzable substance(s) was found in homoge-
genates prepared from the lungs of BCG vaccinated
guinea pigs which inhibited the endogenous
respiration of virulent tubercle bacilli. This
substance(s) also inhibited the increased respiration
of virulent tubercle bacilli produced by
lactate, pyruvate, and homogenates prepared
from the lungs of nonvaccinated guinea pigs.

No inhibition of respiration was obtained with
the sera of vaccinated guinea pigs, nor was
convincing evidence obtained that an inhibitory
substance(s) was present in the spleen or liver
homogenates.

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