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 1. Aided in part by research grant E-1636 from the National Institutes of Health, U. S. Public Health Service, and in part by a research grant from the Tuberculosis Institute of Chicago and Cook County.

2 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 = <0.001).

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>
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<th>Nonvaccinated</th>
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* 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 Q₀ values between 0.04 and 0.08.

The Q₀ values of the endogenous respiration of the bacterial cells varied between 0.46 and 1.10; most of the values were around 0.60.
Figure 1. Effect of three homogenates (A, B, and C) on the endogenous respiration of Mycobacterium tuberculosis.

Figure 2. Effect, expressed in terms of per cent increase or decrease of the endogenous respiration, of homogenates prepared from lungs of vaccinated and nonvaccinated guinea pigs on the respiration of Mycobacterium tuberculosis.

greater than the endogenous for 1½ to 2 hr, only to be less than the endogenous for the rest of the experiment. The $Q_{O_2}$ 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 homoge-
nates 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 homoge-
nates from vaccinated animals to inhibit respi-
ration.

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 nonvacci-
nated guinea pigs. The homogenate which inhib-
ited the endogenous respirations of the mycobac-
terial 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 nonvacci-
nated 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 respira-
tion of virulent tubercle bacilli. The detection of
the substance(s) in the lungs of guinea pigs which
inhibits respiration of tubercle bacilli is compli-
cated by the release in the homogenization
process of substances which increase the oxygen
uptake. The ratio between the amount of respira-
tion increasing and respiration inhibiting sub-
stance(s) will determine the rate of oxygen up-
take. Only when the amount of inhibitor present
is sufficient to completely counteract the respira-
tion 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 rela-
tion to the mechanism of acquired immunity in
tuberculosis. The capacity of the lungs to inhibit
the respiration of tubercle bacilli may have de-
veloped only coincidentally with other changes
more significantly related to increased resistance
to infection. On the other hand, the demonstra-
tion 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 characteris-
tically occurs in the tissues of immunized animals,
especially when the complete dependence of
tubercle bacilli on molecular oxygen for respira-
tion is taken into consideration. A specifically
acquired capacity of tissue to inhibit the respira-
tion of virulent microorganisms would constitute
a unique immune mechanism. However, a role
for the respiration-inhibiting factor(s) in im-
munity to tuberculosis can be postulated only
when it can be shown that increased resistance to
tuberculous infection will follow the administra-
tion of this substance(s) to normal nonim-
munized 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-increasing 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 nondialyzable substance(s) was found in homogenates 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.

REFERENCES


PAGEL, W. 1935 The bactericidal power of the blood serum as a means of differentiating a certain type of pulmonary tuberculosis. Tubercle, 16, 256.


