INSTRUCTION OF ACQUIRED RESISTANCE IN GUINEA PIGS WITH DEFATTED MYCOBACTERIUM TUBERCULOSIS VACCINES


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ABSTRACT

SMITH, D. W. (University of Wisconsin, Madison), G. B. FREGNAN, L. DELAQUERIERE-RICHARDSON, and E. VALDIVIA. Induction of acquired resistance in guinea pigs with defatted Mycobacterium tuberculosis vaccines. J. Bacteriol. 88:87–92. 1964.—Residues of mycobacteria exhaustively extracted with neutral organic solvents have been shown to produce a level of resistance against tuberculosis in guinea pigs comparable to that produced by BCG. Disruption of defatted tubercle bacilli in adjuvant eliminated the acid-fast staining property of the residue, but immunizing and sensitizing properties were retained in the fraction which sedimented at 44,000 × g. To demonstrate these activities with 50 μg of vaccine, it was necessary to use Bayol-Arlacel adjuvant. A similar quantity of vaccine given in saline was inactive. A defatted vaccine prepared from a virulent strain of Mycobacterium tuberculosis recently passed through a guinea pig was significantly more immunogenic than a similar vaccine prepared from H37Ra.

The development of resistance against tuberculosis in guinea pigs vaccinated with defatted tubercle bacilli was reported in earlier publications from this laboratory [see Fregnan and Smith (1963) for a more complete bibliography] as well as from other laboratories (Weiss and Wells, 1960; Tamura et al., 1960). Contrary findings have been published by several investigators (Raffel, 1946; Frappier, Portelance, and St. Pierre, 1950). This paper presents the results of experiments designed to further fractionate defatted tubercle bacilli with the aim of eliminating inert substances and, if possible, bringing about the separation of the factors responsible for the induction of resistance and delayed sensitivity.

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MATERIALS AND METHODS

Animals. Male and female guinea pigs, primarily albino but some mixed colors, weighing 500 to 800 g were obtained from a local supplier. They were fed standard guinea pig chow and were water-supplemented daily with vitamin C (160 μg/ml). Guinea pigs were randomly allocated to 15 groups, each containing 18 animals, and were housed in groups of 2 to 4 in stainless-steel cages.

Strain of Mycobacterium. Most of the defatted vaccines and the challenge inoculum were prepared from a strain of Mycobacterium tuberculosis (NIH-199RB) obtained from B. J. Olson in 1947, and maintained since that time by semiannual transfers on American Type Culture Society medium. One defatted vaccine was prepared from a culture of H37Ra obtained from E. H. Runyon in 1956 and maintained as indicated above.

Preparations of defatted bacillus (DFB) vaccine. A brief description of the various vaccines used in this experiment is given; additional details about each preparation will be supplied on request. Since these substances are crude cell residues that will require further fractionation, no chemical analysis is given at this time.

DFB-H37Ra vaccine was prepared from a 10-day-old culture of H37Ra in Dubos broth (no albumin) with 1.5% glycerol. The live cells were extracted sequentially with ether-ethanol, chloroform, and methanol-chloroform. The final residue was acid-fast and nonviable.

DFB-199GP vaccine was prepared from a culture of M. tuberculosis 199RB freshly isolated from a guinea pig. Other details of preparation are the same as described for DFB-H37Ra.

The preparation of lot 3 of DFB vaccine was described previously (Fregnan and Smith, 1963).

Vaccines derived from the disruption of DFB lot 3. The essential steps in the preparation of various vaccines from DFB lot 3 are summarized in Fig. 1. Disintegration was carried out in a Nossal (1953) apparatus. The capsule, cooled by
a stream of compressed CO₂, was charged with 10 ml of adjuvant (Bayol-Arlacel; 85:15, v/v) or distilled water, 14 g of Pavement Making Beads (Minnesota Mining and Mfg. Co., St. Paul, Minn.), and 250 mg of DFB lot 3. The apparatus was stopped at 3-min intervals to observe the temperature of the capsule.

BCG. Lyophilized BCG vaccine (supplied by the Tice Laboratory of the Institution for Tuberculosis Research, affiliated with the University of Illinois and Research Foundation, Chicago, Ill.) lot 9498-79 was resuspended in diluent to a final concentration of 1 mg/ml. Each animal in the group received 0.1 ml of vaccine by the subcutaneous route.

Vaccination procedure. With the exception of one group of guinea pigs vaccinated with DFB-AR (50 μg in 0.1 ml of saline by the intraperitoneal route), all vaccines containing DFB residues were given subcutaneously in theinguinal region and were suspended in adjuvant (0.5 mg/ml). Adjuvant-soluble fractions were given directly as the supernatant fraction of the final centrifugation. With the exception of BCG (given once only), all vaccines were given two times 3 weeks apart in 0.1 ml of suspension containing 50 μg of vaccine.

Animals in the control group were inoculated with 0.1 ml of adjuvant at the time of vaccination of the other groups.

Tuberculin tests. All groups were tuberculin-tested 3 days before the second vaccination and approximately 3 months later at a period 3 days before challenge. Skin tests were made with 250 tuberculin units of purified protein derivative given intradermally in 0.1 ml of buffered diluent. Observations of the skin test sites were made at 24 and 48 hr.

Challenge. The challenge suspension was prepared from a 9-day-old culture of M. tuberculosis 199RB, isolated from a mouse spleen and in its second passage in Tween-albumin medium. The culture was diluted to 10⁻³ in saline containing one part in ten of Tween-albumin medium. Plate counts made on Dubos oleic acid albumin agar (Difco) indicated that each animal received approximately 10⁵ viable units. The challenge culture was maintained in uniform suspension by a magnetic stirrer during the infection procedure. Each animal received 1.0 ml of suspension subcutaneously over the sternum 5 months after the first vaccination.

Autopsy procedure. Six weeks after challenge, the guinea pigs were killed by the intraperitoneal injection of 2.0 ml of pentobarbital sodium (60 mg/ml). Observations were made in the following sequence: body weight, axillary and inguinal lymph nodes, tracheobronchial and retrosternal nodes, lung, liver, spleen, and spleen weight. The tracheobronchial nodes, lungs, and heart were fixed as one aggregate of tissue. The extent of gross tuberculosis was recorded according to a modification of the Feldman (1943) index. The maximal modified score was 90, subdivided as follows: for spleen involvement, the range was 0 to 35; for lung, 0 to 30; and for the liver, 0 to 25. Observations on each animal taken at random from the different groups were made by one person without knowledge of the group from which the animal had been taken.

Histopathological observations. After formalin fixation, sections were cut from segments of the spleen, liver, lungs, inoculation site, and tracheobronchial nodes. Sections were stained routinely with hematoxylin and eosin. Duplicates of some slides were stained with Ziehl-Neelsen stain. Each slide was studied microscopically by an independent observer, and the extent of tuberculous lesions was recorded according to a modified Feldman index, the maximal score allotted each organ being the same as for gross lesions (see above).

RESULTS

Figure 1 illustrates the main steps in the preparation of the subfractions of DFB lot 3. The other vaccines included in the experiment are grouped at the bottom of the figure. The results obtained with each vaccine are indicated as the total Feldman index over the diameter of the tuberculin reaction in millimeters. It can be seen that some of the fractions have greater immunizing potency than DFB lot 3, whereas others are inferior. Vaccines derived from alkaline ethanol-extracted DFB were definitely less potent whether Nossalized in adjuvant or in water. DFB-AR was again shown to be at least as potent as DFB when given in adjuvant by the subcutaneous route, but the same quantity of vaccine given by the intraperitoneal route was devoid of immunogenicity or allergenicity. Digestion of DFB-AR with ribonuclease, pepsin, and trypsin did not result in a significant loss of activity. It is evident that the substances which were released
**DFB-Lot 3**

15.5

11

- Extracted with alkaline ethanol 48 hr at 37°C
- Residue nossalized 9 min in adjuvant or water

Water residue  Adjuvant residue

- Centrifuged at 44,000 x g
- Digested with ribonuclease, trypsin, and pepsin
- Centrifuged at 822 x g
- Residue lyophilized

**DFB-AR**

- Given IP in saline
- Given SC

Ref: DFB-AR

- Residue
- Supernatant

Supernatant  Residue

**DFB-AR**

- Digested with 44,000 x g
- 49.7

3

**DFB-AR**

- Residue
- Supernatant

Supernatant  Residue

**DFB-AR**

- 105,000 x g

105,000

**DFB-AR**

105

**DFB-AR**

9.5

11

**DFB-AR**

48.7

4

*Mean of the Feldman index (total) over the mean of the diameter of the tuberculin reaction in millimeters (evaluated 3 days before challenge).

DFB-H37Ra - 18.6

13

DFB-199GP - 6.0

15

BCG - 9.2

9

Nonevaccinated - 53.8

0

**Fig. 1. Flow diagram giving the steps in preparation of subfractions of DFB.**
from DFB by disintegration in adjuvant and which did not sediment at 44,000 × g possessed neither immunizing nor hypersensitizing activity. A further separation of the adjuvant-extracted material by centrifugation at 105,000 × g yielded a very small amount of residue with an intermediate level of activity and a supernatant fraction devoid of either immunizing or hypersensitizing activity.

The final group of fractions was prepared from DFB lot 3 in an attempt to obtain a residue as free as possible from the adjuvant-soluble fraction. This was accomplished by interrupting the disintegration process at 3-min intervals, separating the residue by centrifugation, and returning it to the Nossal capsule with fresh adjuvant. This process was repeated three times. The final residue was washed with hexane and water, and vaccines were prepared from the water-soluble fraction and the final residue. The results indicate that the water-soluble fraction, DFB-AR-Water Soluble, was devoid of immunizing or hypersensitizing activity, whereas the residue, DFB-AR-Purified, possessed the same activity as DFB-AR.

The most potent vaccine seen in this experiment was DFB-199GP, the preparation of DFB derived from a fresh guinea pig passage of M. tuberculosis 199RB. In contrast, the DFB preparation made from H37Ra, DFB-H37Ra, was significantly less immunogenic. BCG induced a level of acquired resistance equivalent to DFB-AR, but with a lower level of sensitivity.

In Table 1 are presented data comparing the means of the gross and microscopic spleen index, gross and microscopic total Feldman index, and the tuberculin test results. The vaccine groups are arranged in the order of increasing extent of gross tuberculosis. We previously reported (Fregnan and Smith, 1963) a correlation between the index for the spleen and the index for the whole animal; this relationship is also seen in the results of this experiment. A very good correlation is also seen between the independent evaluations made of the gross and microscopic Feldman index. Granulomatous lesions and necrosis were seen in all groups. The most striking histopathological difference between the groups having the highest and the lowest mean Feldman index was in the number and size of the lesions, and in their degree of confluence and of central necrosis. There was no clear-cut image of scarred tuberculous lesions in any of the slides examined.

<table>
<thead>
<tr>
<th>Vaccine</th>
<th>Spleen index</th>
<th>Total index</th>
<th>Tuberculin test</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Gross</td>
<td>Microscopic</td>
<td>Gross</td>
</tr>
<tr>
<td>DFB-199GP</td>
<td>2.9</td>
<td>3.1</td>
<td>6.0</td>
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<td>BCG</td>
<td>5.2</td>
<td>6.2</td>
<td>9.2</td>
</tr>
<tr>
<td>DFB-AR</td>
<td>5.6</td>
<td>6.7</td>
<td>9.5</td>
</tr>
<tr>
<td>DFB-AR-Purified</td>
<td>4.2</td>
<td>6.5</td>
<td>9.5</td>
</tr>
<tr>
<td>DFB-AR-Digest</td>
<td>7.6</td>
<td>7.8</td>
<td>15.2</td>
</tr>
<tr>
<td>DFB-Lot 3</td>
<td>7.6</td>
<td>10.5</td>
<td>15.5</td>
</tr>
<tr>
<td>DFB-H37Ra</td>
<td>10.6</td>
<td>12.6</td>
<td>18.6</td>
</tr>
<tr>
<td>DFB-AR-105,000</td>
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<td>19.4</td>
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<td>25.0</td>
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<tr>
<td>DFB-AS-105,000</td>
<td>28.2</td>
<td>29.0</td>
<td>60.6</td>
</tr>
</tbody>
</table>

* Average diameter of erythema and induration 48 hr after intradermal inoculation of 250 tuberculin units of purified protein derivative.
DISCUSSION

In this paper, attempts to increase the immunizing potency of defatted tubercle bacilli are reported. Separation of the immunizing and hypersensitizing constituents was also attempted. The only available assay for measuring these two biological activities is the immunization and challenge of experimental animals. Guinea pigs were chosen to make a simultaneous study of immunogenic and allergenic potencies of the various vaccines. A challenge-sacrifice type of evaluation was chosen for reasons given earlier (Fregnan and Smith, 1963).

Loss of acid-fast staining property was used as evidence that a change had been brought about in the DFB vaccine and was noted after disintegration in the Nossal apparatus and after extraction with alkaline ethanol. Several investigators have employed the acid-fast stain to determine the degree of contamination of cell walls of mycobacteria by relatively intact acid-fast bacilli (Kotani et al., 1960; Kanai, 1962). Electron microscope studies of some of the adjuvant residue vaccines used in these experiments suggest that protoplasmic constituents adherent to cell-wall fragments are present.

One of the extraction procedures employed in this study in an attempt to bring about a further resolution of the mixture of components of DFB was the method of Cummins and Harris (1958). This procedure involves extraction with alkaline ethanol, followed by disintegration and enzyme digestion. In a previous report (Erikson and Smith, 1962), it was shown that DFB extracted with alkaline ethanol lost its acid-fast character but retained its immunogenicity for guinea pigs. In the current work, alkaline ethanol-extracted DFB was subjected to Nossal disintegration either in adjuvant or in water, and the resulting residues were digested with ribonuclease, pepsin, and trypsin. A reduction of immunogenicity and allergenicity was noted for both preparations. The loss of sensitizing activity for the residue of alkaline ethanol-extracted cells disintegrated in this manner was similar to the results obtained by Kanai, Youmans, and Youmans (1960) with alkaline ethanol-extracted cell walls.

In a previous experiment (Fregnan and Smith, 1963), it was shown that DFB disrupted in adjuvant was more immunogenic than DFB disrupted in saline. Similar findings were reported recently by Larson et al. (1963), who disrupted BCG cells in oil and in saline in a Sorvall-Ribi refrigerated cell fractionator. They concluded that the oil-insoluble disruption products were highly immunogenic in mice, whereas fractions prepared from BCG cells disrupted in water were inactive. In earlier experiments from this laboratory (Fregnan and Smith, 1963), some immunogenicity was detected in the fraction remaining in the adjuvant supernatant fluid after centrifugation at 44,000 × g; therefore, an attempt was made in the current experiment to obtain a more clear-cut separation of adjuvant-soluble and -insoluble fractions. This was accomplished by repeated centrifugation of the products of disintegration at 44,000 × g followed by centrifugation at 105,000 × g. Immunogenicity

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>Degrees of freedom</th>
<th>Sum of squares</th>
<th>Mean square</th>
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<tr>
<td>Total</td>
<td>240</td>
<td>1670.515780</td>
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<tr>
<td>Among groups</td>
<td>14</td>
<td>864.805731</td>
<td>61.771838</td>
<td>17.327**</td>
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<tr>
<td>Control versus others</td>
<td>1</td>
<td>113.529452</td>
<td>113.529452</td>
<td>31.845**</td>
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<td>Others among selves</td>
<td>13</td>
<td>751.276279</td>
<td>57.790483</td>
<td>16.210**</td>
</tr>
<tr>
<td>Within groups</td>
<td>226</td>
<td>805.710049</td>
<td>3.565089</td>
<td></td>
</tr>
</tbody>
</table>

Coefficient of variation = 39.32.

<table>
<thead>
<tr>
<th>Vaccine</th>
<th>No. of animals</th>
<th>Feldman index</th>
</tr>
</thead>
<tbody>
<tr>
<td>DFB-199GP</td>
<td>17</td>
<td>3.8</td>
</tr>
<tr>
<td>BCG</td>
<td>16</td>
<td>6.5</td>
</tr>
<tr>
<td>DFB-AR</td>
<td>17</td>
<td>6.5</td>
</tr>
<tr>
<td>DFB-AR-Purified</td>
<td>17</td>
<td>7.7</td>
</tr>
<tr>
<td>DFB-AR-Digest</td>
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<td>10.9</td>
</tr>
<tr>
<td>DFB-Lot 3</td>
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<td>11.9</td>
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<tr>
<td>DFB-H37Ra</td>
<td>16</td>
<td>16.4</td>
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<td>DFB-AR-105,000</td>
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<td>DFB-AE-WR</td>
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<td>39.2</td>
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<td>43.9</td>
</tr>
<tr>
<td>DFB-AR-Water Soluble</td>
<td>15</td>
<td>45.6</td>
</tr>
<tr>
<td>DFB-AR (ip in saline)</td>
<td>13</td>
<td>46.3</td>
</tr>
<tr>
<td>Control</td>
<td>17</td>
<td>52.7</td>
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<tr>
<td>DFB-AS-105,000</td>
<td>17</td>
<td>58.0</td>
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was detected only in the residue separated from DFB by centrifugation at 44,000 × g.

It should be noted that while 50 μg of DFB-AR given subcutaneously in adjuvant produced high levels of immunity and sensitivity, the same quantity given by the intraperitoneal route in saline was devoid of activity.

DFB vaccines prepared from an avirulent strain of mycobacterium (H37Ra) and from a recently passed virulent strain gave significantly different levels of protection, whereas levels of hypersensitivity were the same. Further studies will be required to confirm this observation and to determine whether such differences can be obtained for the residues of these two vaccines after disruption.

Comparison of the mean immunogenic and allergenic potencies of the various substances tested in these experiments gives no evidence that different fractions are responsible for the two activities. In fact, with the exception of BCG and DFB-H37Ra, a good correlation exists between the mean immunogenic and allergenic activities of the various vaccines.

Acknowledgments

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Literature Cited


