Comparison of Extracts of Tubercle Bacilli and BCG as Immunizing Agents in Guinea Pigs.* (22118)

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Recent investigators studying biological properties of fractions of tubercle bacilli have for the most part used the mouse for *in vivo* studies. Bloch and Noll(1) reported that tuberculosis in black mice of Strain C57 is enhanced by administration of cord factor. Weiss and Dubos(2) report that a fraction extracted from *Mycobacterium tuberculosis* by absolute methanol is capable of eliciting in mice a state of increased resistance to infection.

Notwithstanding these contributions based on a mouse evaluation of biological properties of fractions and extracts of the tubercle bacillus, we wish to report studies carried out with guinea pigs for *in vivo* determinations of immunizing activity of extracts of mycobacteria. Accordingly, we present results of experiments concerning immunizing activity in guinea pigs of extracts of non-heat killed tubercle bacilli.

Methods and materials. Most fractionation studies reported at this time were made using *Mycobacterium tuberculosis var. hominis* NIH strain 199RB. This culture has been used since the inception of this research in 1946.‡ Studies of strains more recently isolated from sputum are currently being made in an attempt to find a culture possessing greater immunizing potency than does 199RB. Variations were made in procedure for growth and fractionation of cultures from one experiment to another, to evaluate critically the conditions necessary for isolation of the most potent fraction; however, procedures in general were as follows. Mass cultures were prepared on modified Long's medium(3) made up in 25 liter quantities and distributed into one-gallon jugs, each one containing 900 ml. The inoculum for 30-60 such cultures, usually a 2-week-old surface growth, was taken from one 900 ml culture flask. The cultures were subsequently incubated at 37°C for 3 to 12 weeks. After incubation, the culture fluid was withdrawn into 5 gallon bottles containing Cresylone§ and mineral oil (antifoam), autoclaved and discarded. The culture pellicle from harvested jugs was combined, by means of a vacuum system, into one container which was then completely filled with the first extracting solvent (usually ethanol) and allowed to stand 48 hours. This step can usually be considered the point at which the cells become nonviable. Following ethanol treatment, the residue was extracted 24-48 hours with ethanol-ether (50-50), methanol-chloroform (50-50), or chloroform.

Preliminary separation of the extracted components was occasionally made by cooling the methanol-chloroform solution first to 5°C and collecting a 5°C insoluble fraction. The filtrate from this separation was cooled to −20°C at which temperature a second insoluble residue was collected. The filtrate upon evaporation deposits a residue designated the −20°C soluble fraction. Further separations of total extracts or low temperature insoluble fractions were made by column chromatography. For experiments here reported, final separations were made on 50 mm diameter chromatographic columns packed with 85 g of Magnesol‖-Celite¶ and charged with 2 g of

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§ Cresylone- tri-cresol disinfectant of Parke-Davis and Co.

‖ Magnesol—Magnesium Silicate, Product of Westvaco Chemical Co., S. Charleston, W. Va.

¶ Celite—Filter Aid, Johns Manville Co.

the substance to be separated. The columns were developed as adsorption-elution chromatograms with the following series of solvents; petroleum-ether (60-68°C boiling range), petroleum ether-benzene (50-50) benzene, benzene-diethyl ether (50-50) and various mixtures of methanol and chloroform. Infrared spectra were recorded for all extracts and chromatograph-eluted components for purposes of identification of the substance and as a control on reproducibility of the fractionation (Randall *et al.* 4-7). Guinea pigs weighing approximately 500 g were vaccinated with the lipid fractions dissolved in a mixture of 9 parts mineral oil and one part Arlacel A.** Bacillary residues and protein extracts were emulsified from aqueous suspension in the same adjuvant according to the method of Salk(8). As one of the controls, BCG was routinely administered in aqueous suspension either from cultures grown 8 days on liquid Tween-albumin medium or from lyophilized vaccine kindly supplied by Tice Laboratories through the courtesy of Dr. Sol. R. Rosenthal. All animals were vaccinated subcutaneously in the inguinal region or over the sternum. The protocol for each evaluation of bacillary extracts *in vivo* was changed from time to time but generally guinea pigs were vaccinated with 0.1 mg of extract or 1.0 mg of BCG. Three weeks after the second vaccination test guinea pigs and a suitable number of controls, treated with adjuvant alone, were shaved and tuberculin tested with 0.0002 mg of PPD by intracutaneous route. The results were read at 24 and 48 hours, a positive reaction being at least 5 mm minimum diameter. In many instances, reactions were strong with a blanched, later necrotic, center. Following the tuberculin test, all groups were randomized and challenged. The 18-20 guinea pigs in each group were housed in 3 or 4 stainless-steel cages. For randomization, the 3 or 4 cages in any one experimental group were redistributed at random so that one cage would be found in the first quarter of the total number, a second cage in the second quarter of the total and so on. The code for the newly assigned cage numbers was sealed and not examined until post mortem examination of all groups had been completed. The following advantages were presumed to result from randomization. It would tend to minimize the possibility that variations in challenge suspension or procedure would affect any one experimental group, and would also reduce unintentional bias which might be shown in evaluating the extent of gross disease at autopsy. During post mortem examination, each successive series of 5 animals has come from a different treatment group. This results in a constant change in extent of gross tuberculosis and would discourage any inadvertent attempt to reduce the variation from one group to the next.

The challenge suspension was prepared from an 8-day culture of strain 199RB on liquid Tween-albumin medium. The culture was first filtered through Whatman #1 filter paper, adjusted to 85% transmission at 500 μ (Coleman Junior spectrophotometer) and then diluted to 10^{-5} with saline containing 0.5% bovine albumin fraction V. Plate counts made of this challenge suspension contained 300-1000 viable units. The challenge was given subcutaneously in 1 ml of diluent in the inguinal region or over the sternum. Six weeks after challenge the guinea pigs were sacrificed (ether) and the extent of gross tuberculosis in the viscera estimated according to the scheme of Feldman(9). It is important to state that gross evaluation of the lungs was difficult to make in early experiments because of intercurrent infection with group "C" streptococcus, however it has been our experience with strain 199RB that the spleen is a good index of the extent of tuberculosis in the guinea pig. In order for this to be true, the challenge should be such that some animals will have minimal or no gross disease. If the spleen is extensively involved, showing enlargement and confluent tubercles, one expects to find disease in the lungs and liver. Conversely, if there is no splenic involvement, little disease is seen in other organs. Observations were also made on the site of inoculation, as well as inguinal, tracheobronchial, and retrosternal lymph nodes. The

** Arlacel A—Product of Atlas Powder Co., Wilmington, Del.

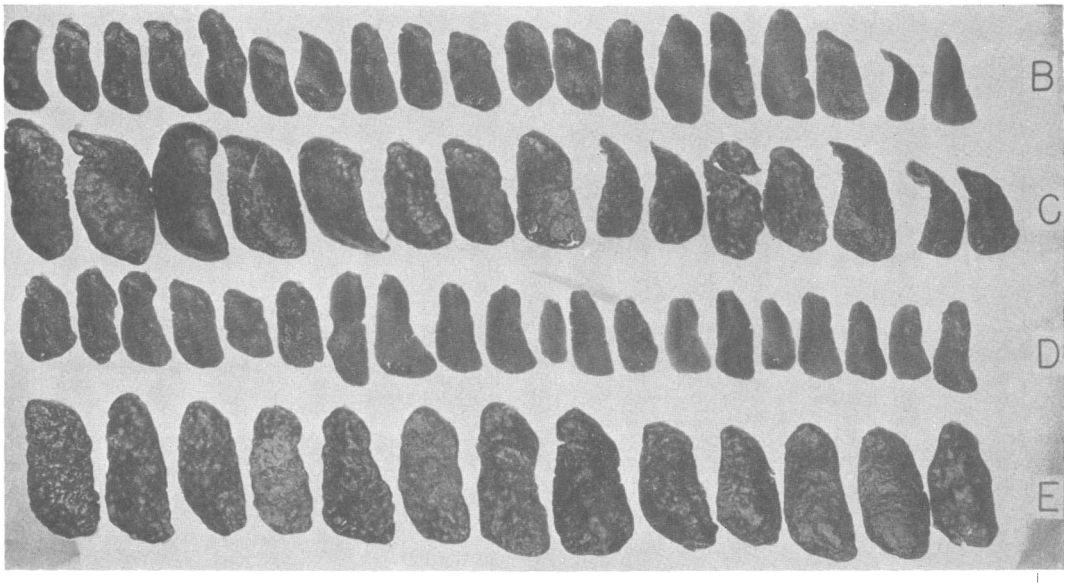

FIG. 1. Photograph of formalin fixed spleens from 4 groups of animals taken from the experiment recorded in Table IV.

Group B—vaccinated with 0.05 mg of defatted bacilli
" C—alcohol-ether soluble fraction
" D—1.0 mg of lyophilized BCG
" E—non-vaccinated controls

Spleens are arranged in approximate order of decreasing amount of gross disease reading and numbering from left to right. Typical Feldman Index values for spleen are as follows. Group B—First 4 spleens, value 20; spleens 5, 6 and 7, value 15; spleens 8 through 13, value 10; spleens 14 through 17, value 5; and spleens 18 and 19, value 0. Group C—All spleens given value of 35 with exception of last 2 which were recorded as 30 and 25 respectively. Group D—Spleen 1, value 20; spleen 2 through 5, value 15; spleens 6 and 7, value 10; spleens 8 through 12, value 5; and spleens 13 through 20, value 0. Group E—Spleens 1 through 18 recorded as 35; spleen 19 recorded as 30 (this group was reduced for photographic purposes by leaving out 6 spleens between spleens 1 and 2).

extent of gross disease was recorded according to the scheme of Feldman, slightly modified. A value of 5-10 was recorded for lungs on which less than 12 surface lesions could be counted. For greater than 12 lesions, values of 15 and 20 were assigned and when the number of lesions was too large to count, values of 25 or 30 were given depending upon the over-all appearance. By referring to Fig. 1, one can see numerical assignments typical of those made for spleen involvement.

Results. The purpose of the first experiment was examination of 3 variables, namely, age of culture to be extracted, concentration of ethanol used in the first cell treatment, and use of alpha tocopherol acetate as an antioxidant, for their effects on immunizing potency of a crude chloroform extract. Ninety bottles of modified Long's medium were inoculated with culture 199RB. Sixty were harvested after 3 weeks incubation, and the remaining 30 after 12 weeks incubation. Half of the cultures at each period were treated with 50% ethanol and half with 95% ethanol. A like division of cell mass was made for subsequent extraction with chloroform containing tocopherol or with reagent grade chloroform. This series of variables ultimately gave rise to the following eight crude lipid extracts: 1) 3W50T—extract of 3 week (3W) culture treated with 50% ethanol (50) and extracted with chloroform containing tocopherol (T), 2) 12W95—extract of 12 week culture (12W) treated with 95% ethanol (95) and extracted with chloroform, 3) 3W50, 3W95, 3W95T, 12W50, 12W95T, 12W50T.

Several observations may be made from the results of this experiment recorded in

TABLE I. Feldman Index Values for Immunized and Control Guinea Pigs Sacrificed 6 Weeks after Challenge.

Vaccine	No. of G. pigs sacrificed	Tuberculin reaction‡	Mean Feldman index§
BCG*	18	—	26‖
3W50T†	16	+	40
3W50	16	+	40
3W95T	16	—	58‖
3W95	19	—	51
12W50T	16	+	44
12W50	18	+	36
12W95T	18	—	47
12W95	19	—	32¶
Control	19	—	43

* Lyophilized vaccine (Tice 968-P), 0.5 mg given twice 3 wk apart.

† All lipid fractions suspended in mineral oil containing 1 part in 20 of aquaphor (1 mg of lipid in 0.1 cc of adjuvant) in two doses 3 wk apart.

‡ Tuberculin test, 0.0002 mg of PPD intracutaneously, read at 24 and 48 hr.

§ Three wk after second vaccination all groups were randomized and challenged with 800 viable units (plate count) given in 1 cc intraperitoneally. All groups were sacrificed 6 wk after infection and extent of gross disease recorded according to the scheme of Feldman.

Autopsy index:

	Spleen	Lung	Liver
Extensive	35	30	25
Moderate	20	20	20
Slight	10	10	10

(Moderate lung 12 or more discrete tubercles, slight 2-11 tubercles given value of 10, 1 tubercle value of 5.)

‖ Significant at the 1% level.
¶ " " " 5% " .

Table I. There appears to be little or no immunizing value in any extract of 3-week-old cultures while at least one extract of the 12-week culture appeared to give some protection. Extracts prepared from 50% ethanol treated cultures uniformly caused the development of a high level of tuberculin hypersensitivity†† whereas no hypersensitivity developed in animals vaccinated with extracts from 95% ethanol treated bacilli or BCG. One extract (3W95T) appeared to lower the resistance of guinea pigs to subsequent infection with tubercle bacilli.

Extracts evaluated in the second test were modifications of the three substances which showed some evidence of activity in the first experiment. Two extracts were precipitated at −20°C from methanol-chloroform yielding −20°C soluble fractions (−20S) and −20°C insoluble fractions (−20I). A third extract was chromatographed on a Magnesol-Celite column packed with 85 g of adsorbent. The column was charged with 2 g of the 12W50 extract and eluted with 1800 ml amounts of the following series of solvents: petroleum ether (PE), petroleum ether-benzene (PEB), benzene (B), benzene-ether (BE), methanol (M) and chloroform (C). The hold-back volume (volume of solvent retained by the packed column) for the column was approximately 300 ml and eluates were collected as complete hold-back volumes. Methanol eluates were divided into chloroform soluble and chloroform insoluble parts.

It was not possible to demonstrate resistance lowering activity with any preparation

TABLE II. Feldman Index Values for Immunized and Control Guinea Pigs Sacrificed Six Weeks after Challenge.

Vaccine	No. of G. pigs sacrificed	Tuberculin reaction‡	Mean Feldman index§
BCG*	12	+—	8‖
12W95† (1 inj.)	17	—	27
12W95	14	—	25¶
" -20I	18	—	20¶
" -20S	14	—	33
3W95T	16	—	26
" -20S	17	—	35
" -20I	14	—	33
12W50	14	—	19‖
" PeB-2	17	—	29
" Pe (5-6)	8	—	36
" PeB-1, Pe-3	15	—	27
" BE-1	13	—	30
" BE-3	15	—	25¶
12W50M_1CHCl_3S	14	—	32
" $CHCl_3I$	15	—	26
Controls	18	—	36

* Fresh vaccine (Phipps strain) 1 mg given one time only with the first injection.

† Lipid fractions suspended in Baylol-Arlacel, 0.1 mg given in 2 doses 3 wk apart.

‡ Tuberculin test, 0.0002 mg PPD intracutaneously, read at 24 and 48 hr.

§ Three wk after second vaccination all groups were randomized and challenged with 700 viable units of strain 199RB subcutaneously in the inguinal region. Sacrificed 6 wk after infection.

‖ Significant at the 1% level.
¶ " " " 5% " .

†† Selected animals from hypersensitive groups were removed before challenge and maintained in separate quarters as non-challenge controls. They were autopsied at the same time as other groups and the tissues were cultured and found to be negative with respect to acid-fast bacilli.

TABLE III. Feldman Index Values for Immunized and Control Guinea Pigs Sacrificed Six Weeks after Challenge.

Vaccine	No. of G. pigs sacrificed	Tuberculin reaction‡	Mean Feldman index§
BCG (1 mg)*	14	+	11
12W50 (.1 mg)	20	+	24
" (1 mg)	19	+	15
" (2 mg)*	17	+	16
" -5I	18	±	22
" -20I	20	+	21
" -20S	15	—	32
" -column	17	—	36
" -column + defatted bacilli†	17	+	9
Defatted bacilli†	15	+	12
Controls	19	—	37

* Given one time only at the first inoculation (BCG fresh Phipps strain).

† Defatted bacilli were suspended in adjuvant so that each 0.1 cc would contain 0.05 mg of cells (dry wt). Unless stated otherwise all lipid fractions were given in adjuvant (0.1 mg in 0.1 cc).

‡ Tuberculin test, 0.0002 mg of PPD, read at 24 and 48 hr.

§ Three wk after second vaccination all groups were randomized and challenged with 800 viable units of strain 199RB in 1 cc of saline. The cultures were filtered through Whatman #1 filter paper and 10 cc syringes were used once only. The last 1 cc volume in each syringe was discarded. Six wk after infection all groups were sacrificed and the extent of gross tuberculosis recorded according to the scheme of Feldman.

in this test. From the Feldman index figures it appears that a significant level of resistance was stimulated only in groups vaccinated with BCG and to a lesser extent with 12W50 and 12W95-20I. However, no fraction separated from 12W50 by column chromatography possessed the level of activity found in the parent fraction. It is interesting to note that fraction 12W50 Pe(5-6) was later identified by infrared spectroscopy as a contaminating oil inadvertently introduced in one of the extraction steps. From this experiment it is to be concluded that an active immunizing component was not separated from 12W50 by chromatography. Among possibilities which may account for this failure are the following: to produce an immunizing effect in the guinea pig, several components separated on the column would have to be combined, or activity of the fraction is destroyed on the chromatographic column, or finally that the active material is not eluted from the column.

In the experiment in Table III a new column was prepared and the 12W50 fraction applied in the same manner. The column was developed with the same series of solvents, however, this time all eluates were collected in one receiver and evaporated to dryness. The residue collected in this manner was tested as such and also after combining it with bacillary residue remaining after neutral solvent extraction (defatted bacilli). In addition, the 12W50 fraction was precipitated at 5°C and –20°C.

Comparing the results of this experiment with the previous one, it can be seen that approximately the same level of immunizing activity was obtained with 0.1 mg amounts of the 12W50 fraction, the chloroform soluble fraction of 50% ethanol treated bacilli. When the amount of fraction injected was increased to 1 mg in two injections or 2 mg in one injection, a higher degree of resistance to infection was produced. Fractions precipitated at 5°C and –20°C appeared to contain all the activity of the parent fraction, but no increase in activity was noted. The pool of eluates from chromatographic separation of 12W50 had completely lost all the original activity, however, when mixed with defatted bacillary residue, significant increase in potency of the preparation was noted. Defatted bacilli in adjuvant produced an increased resistance which compares favorably with some of the best preparations thus far evaluated. Finally it should be noted that the most significant protection was produced in animals that had been rendered hypersensitive to the extent that they gave positive tuberculin reactions with 0.0002 mg of PPD.

The last experiment is one in which further investigations of the defatted bacilli were made. Determinations were made of the effect of heat (121°C for 20 min.), extraction with borate buffer and the effect of further extraction of the residue with alcohol-ether containing 3% HCl.

From the results, Table IV, it would appear that extraction of neutral solvent insoluble residue with alcohol-ether containing 3% HCl destroys the remaining activity of this substance, since neither the soluble portion,

Anderson's(10) "firmly bound lipids," nor the residue after this extraction, possessed any ability to increase resistance of guinea pigs to tuberculosis. In addition it appears that 0.05 mg of defatted bacilli is the minimal effective amount and that autoclaving does not greatly affect the activity of the preparation. The fraction soluble in borate buffer at pH 9.1 had an intermediate activity which might have resulted from the presence of small numbers of intact cells.

Discussion. One of the major problems encountered in an examination of biological properties of fractions of tubercle bacilli, is removal of intact bacilli from extracts and fractions, Raffel(11), Myrivik(12). Results of an earlier report of this work (Smith *et al.*) (13), in which a smaller number of animals were used, are open to question because filtration was the only method employed to remove intact bacilli from extracts. In this report, the same considerations may apply to loss of activity of the 12W50 extract following chromatography.

Results obtained after vaccinating guinea pigs with small amounts of defatted bacilli in adjuvant show that significant increase in anti-tuberculous resistance has taken place. Guinea pigs vaccinated with whole dead bacilli are not protected to the same extent. These results would imply that the vaccine is improved by removing neutral solvent soluble substances (*i.e.* Cord Factor). In another experiment, not reported here, neutral solvent soluble material of a strain other than 199RB did not significantly alter resistance of guinea pigs when tested under similar conditions.

It has been reported by Anderson(10) that after extracting tubercle bacilli with neutral organic solvents it is possible to remove 10% more lipid by treating the bacilli with alcohol-ether containing 3% HCl. When these so-called "firmly bound lipids" were removed from previously extracted bacilli, neither the soluble portion nor the residue possessed any immunizing activity. Accordingly, it would appear that rupture of lipo-protein bonds which hold the firmly-bound lipids to bacillary protein renders the resulting materials inactive with respect to their resistance inducing properties. It is also interesting to note that at this same stage of fractionation, the property of inducing a state of delayed hypersensitivity was also destroyed.

The fact that purified protein or lipid fractions have the ability to stimulate increased resistance to tuberculosis in mice and guinea pigs is shown by the work of Weiss and Dubos(2), Kropp and Floyd(14), Choucroun (15), and Youmans(16). The present work neither confirms nor refutes these earlier observations.

With limitations on space and animal facilities, it has been necessary to carry out evaluations *in vivo* on a screening basis using minimal numbers of guinea pigs for any one vaccine and a challenge-sacrifice type of experiment. Estimations of the extent of disease at autopsy were made according to the method of Feldman. Statistical studies by analysis of variance technic,‡‡ indicates that, for groups of 14-20 animals, 10 points on the Feldman scale represents a difference which is significant at the 5% level. Since the work is still in a preliminary phase it was felt that only limited microscopic examination of tissues could be made. However, a definite correlation was shown to exist between gross and microscopic appearance of spleens and the extent of gross disease recorded for animals in several groups (BCG, 12W50 and Controls) of the experiment recorded in Table II.

Finally attention should be drawn to results obtained with BCG vaccines in the four experiments reported in this paper. Using approximately 1.0 mg of fresh vaccine (Phipps strain) or lyophilized vaccine (Tice strain), significant protection was afforded by BCG in each test. This is perhaps best shown in Fig. 1 where the spleens of four groups of the experiment recorded in Table IV are arranged in the approximate order of decreasing extent of gross disease reading left to right.

Summary. 1. Comparison has been made in guinea pigs of ability of BCG and various extracts and fractions of the tubercle bacillus to stimulate increased resistance to tuber-

‡‡ Courtesy of the Numerical Analysis Laboratory, University of Wisconsin.

TABLE IV. Feldman Index Values for Immunized and Control Guinea Pigs Sacrificed Six Weeks after Challenge.

Vaccine	No. of G. pigs sacrificed	Tuberculin reaction§	Mean Feldman index‖
Defatted bacilli .01 mg	20	+	36
Idem .05 mg	19	+	17
" 1.0 mg*	18	+	29
DFB heated to 121°C 20 min.	17	+	27
DFB extracted (soluble) in Alc-ether (3% HCl)	17	—	62
Residue of above	20	+—	51
Whole cells heated at 121°C 20 min.	19	+	36
DFB extracted with borate buffer	17	+ (min)	34
BCG*	19	+	11
Controls	19	—	63

* Given one time only at time of first inoculation.
† BCG given one time only 6 wk before challenge. 1 mg of 949BLP. (Lyophilized vaccine, Tice laboratories.)
‡ All fractions given as 0.05 mg in adjuvant unless otherwise indicated.
§ Tuberculin tested with 0.0002 mg of PPD.
‖ All animals infected with 300 viable units of culture 199RB in 1 cc of diluent subcutaneously over the thorax. Undiluted culture was first filtered through Whatman #1 filter paper. To reduce possible variations in challenge inoculum, 10 cc syringes were used only once and the last 1 cc volume of suspension in each syringe was not used.

culosis. 2. Significant resistance was induced by 1 mg of BCG, given subcutaneously, in each of 4 experiments reported. 3. Comparable resistance was induced by 0.1 mg (in adjuvant) of a crude chloroform extract, and also by tubercle bacilli which had been exhaustively extracted with neutral organic solvents. 4. The immunizing potency of such defatted bacilli was completely destroyed by extraction with alcohol-ether containing 3% HCl and only partially by autoclaving for 20 minutes. The ability of defatted bacilli to induce delayed hypersensitivity was also destroyed by removal of firmly bound lipids with alcohol-ether containing HCl.

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