STUDIES ON FRACTIONS OF METHANOL EXTRACTS OF TUBERCLE BACILLI

II. Toxic and Allergenic Properties of Fractions Employed as Antituberculous Vaccine

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In view of the disadvantages encountered in the use of living BCG for antituberculous vaccination, some of which are shared by killed tubercle bacilli, the development of an effective non-living, non-toxic, and non-allergenic vaccine would be considered by most workers to be desirable. It was with this in mind that the toxic and allergenic properties of methanol extract fractions have been investigated.

Experiments recently reported (1) have demonstrated that partially purified fractions of a methanol extract of tubercle bacilli are capable of inducing a marked increase of resistance to experimental tuberculosis in mice. Evidence was presented which suggested that more than one mechanism was involved in this elevation of resistance, and that they differed not only in time of appearance, but possibly in specificity as well. Fraction F I, the most promising raw material for the preparation of a suitable vaccine against tuberculosis, became at least two times more potent when mixed with equal amounts of fraction F II from the same methanol extract. Such a relationship is that which exists between an antigen and adjuvant, and was considered worthy of further investigation.

The several experiments reported here will be described in detail as the results are presented. Working definitions and the broad outline of the studies are presented in the following paragraphs.

Toxicity

Primary toxicity was determined by intracutaneous injection of a test material into guinea pigs, followed by direct observation of the injection site at periods ranging from 1 to 24 hours. Substances known to have primary toxicity such as *Serratia marcescens* endotoxin were used for comparative purposes. Reactions were graded according to the scheme devised for the evaluation of allergic skin reactions outlined in Table I.

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Secondary toxic effects are those which cannot be evaluated at the site of application of the test material. These, for lack of better understanding of their nature, are considered manifestations of a general physiologic depression resulting from the primary toxic effects. One indicator by which this depressed state was estimated was the course of weight gain in 4 to 5 week old mice following intraperitoneal injection. A second criterion was depression of resistance to infections by the administration of a test substance at the time of infective challenge. Dubos and Schaedler, referring to this as "enhancement" of infections, found that killed mycobacterial cells and also methanol extract of tubercle bacilli (2) and pertussis vaccine (3) possessed this capacity. The infective organism used for this part of the study was staphylococcus. The

TABLE I

Key to Grading of Intracutaneous Reactions in Guinea Pigs

Intensity of reaction	Description of site	Usual diameter
		mm.
−	Trace color to very faint pink, flat site	<7
±	Very faint to pale pink, flat site	6–8
+	Faint pink to pink, slight induration	7–10
++	Pale to bright pink, moderate induration	9–12
+++	Pink to livid, marked induration, livid or discolored center	12–15
++++	Pink to livid, intense induration, discolored or necrotic center	>13

data, in terms of individual survival times, are tabulated in order of occurrence of death within each group. In a second table, the same data are entered as *cumulative average* survival times. The *mean cumulative average* (*MCA*) is calculated as an indicator of the group response.[1]

Chemical and some biological similarities between F I and the bacterial endotoxin have been suggested (1). One which can be tested precisely is the potency of the material to combine with and neutralize properdin *in vitro* (5). It was of interest to see if F I and its subfractions shared this ability with bacterial lipopolysaccharides.

Allergenicity

The allergenic activities of the various fractions of methanol extract were appraised in two ways. It was of particular interest that a substance prepared from tubercle

[1] The *cumulative averages* were obtained by calculating the average value of available data upon the death of each successive animal. Thus, the first entry is the individual survival time of the first animal to succumb, the second entry is the average survival time of the first two, the third entry of the group is the average of the first three individual survival times, and so on, until all the data have been collected. The *mean cumulative average* (*MCA*) for the group is obtained by taking the mean of the four cumulative averages in the center of the set. The *MCA* reduces the group response to a single term, and represents a compromise value between the *true mean*, which would require assigning an arbitrary value to survivors, and the *median*, which is an unreliable value in a small number of observations. The method is essentially that introduced by Riley (4) and its application to survival experiments has been discussed in detail in a previous publication (1).

bacilli for use as a vaccine against tuberculosis be free from the more common antigens of tuberculin. This was examined by intracutaneous injections into guinea pigs previously made sensitive to tuberculin by whole killed tubercle bacilli.

Even if a given preparation contained no tuberculin antigens, its own antigenic properties might be of the type which could bestow hypersensitivity, as determined by skin reactions. Although a single aqueous injection by any route would not be likely to sensitize an animal, repeated injections, or a single injection with an effective adjuvant might. Experiments designed to test the effect of repeated injections of aqueous solutions were largely inconclusive and will not be reported. An experiment using a Freund type, "complete" adjuvant, on the other hand, indicated that fraction F I was capable of sensitizing guinea pigs to one or more of its components.

Skin reactions in guinea pigs are recorded in the tables, with notations in two parts: (*a*) diameter of reaction site in millimeters, (*b*) intensity of reaction (*i.e.* color and induration, and possibly diameter) expressed in signs from − to ++++. Table I defines the grading system for all intracutaneous reactions including the toxicity tests.

Adjuvant Activity

It was suggested in the first publication of this series that any of the biological effects of whole killed tubercle bacilli might be the result of several chemically distinct substances working together or against one another (1). The same could be said of the remarkable property of tubercle bacilli to mediate immunological phenomena (6). Any purified substance which will serve in this regard and which is devoid of the toxic and allergenic properties of whole tubercle bacilli will be of outstanding importance in immunology. To date nothing seems to have been found to have equal effectiveness in a complete Freund type adjuvant preparation.

Since the fraction F II from methanol extract was thought to have adjuvant activity when combined with F I into a vaccine preparation (1), it was of interest to see how general this effect might be. The now classical system of Landsteiner and Chase (7), later rendered a one-time exposure method (8), was employed. Hypersensitivity can be induced in guinea pigs to picryl chloride by injection of an emulsion of picrylated homologous erythrocyte stromata, mycobacteria, and paraffin oil. The induction is strictly dependent upon the presence of killed mycobacteria in the injection mixtures. Experiments of this design were undertaken with fractions of methanol extract and an acetone extract to test their capacity to replace mycobacteria in a Freund type adjuvant preparation. In these experiments hypersensitivity was determined by contact dermatitis reactions. Picryl chloride in a drop of olive oil was applied over a patch of clipped skin according to the technique employed by Chase (8). The reactions are recorded as to color and induration, the grade comparing roughly with the description of site and intensity shown in Table I.

Methods and Materials

Tubercle Bacilli.—Through the courtesy of research department of Parke, Davis & Co., killed, vacuum-dried *Mycobacterium tuberculosis* var. *hominis* (H37Rv) germ mass was made available for these studies. Culture methods and further treatment were reported previously.

Methanol Extract (ME).—ME is obtained by continuous extraction with methanol at 55°–60°C. of an acetone "defatted" H37Rv germ mass. For this purpose a special apparatus

was designed. This apparatus and all of the following preparative operations have been previously described (1).

F I, F II, F IV.—Crude fractions of ME are obtained by flocculation upon slow concentration at 45°–50°C. F I, the first flocculate, is slightly soluble in petroleum ether [30°–60°], while F II, the second crude flocculate, appears wholly soluble in this solvent mixture. Petroleum ether [30°–60°] is used to further purify F I and F II, serving mainly to separate one from the other. F IV is the final concentrated residue of ME after the removal of fractions F I and F II. Different preparations of these materials are distinguished by a date in parentheses referring to the date of the various methanol extractions. Thus, F I (4/4/57), F I (2/18/58), F I (6/19/58), and F I (11/26/58) were similarly prepared materials, though differing perhaps in relative amounts of the components or relative potencies for some of the activities considered characteristic of F I.[2]

F I-P and F I-S.—Subfractionation of F I yielding these two preparations is effected from water suspensions of F I by the addition of ethanol to 33 per cent and sodium chloride to 0.5 per cent. F I-P, which precipitates under these conditions, represents about 90 per cent of the parent fraction F I. F I-P after washing is resuspended in water. Ethanol is removed from F I-S in the supernatant fluid by evaporation over heat. F I-S is more soluble in a saline solution than distilled water.

Acetone Extract (AE).—Continuous extraction of the tubercle bacilli germ mass for 48 hours at 40°C. constitutes the "defatting" process prior to methanol extraction. Fraction AEP is a copious white, waxy precipitate which forms on cooling acetone extract to room temperature.

Purified Protein Derivative (PPD).—The PPD used in these studies was a Sharp and Dohme, Inc., commercial preparation.

Serratia marcescens Lipopolysaccharide.—The so called endotoxin of *S. marcescens* was a Difco commercial preparation (Control 902148).

Water-in-Oil Emulsions with Adjuvants.—Stable emulsions were prepared from the following ingredients: 2 parts mineral oil (or paraffin oil), 1.1 parts water/oil emulsifier (aquaphor), 1.4 parts aqueous solution. Adjuvant, if killed tubercle bacilli were used, was included in the oil phase before mixing. Antigens were generally included in the aqueous phase. Using a technique described by Chase (9), two Luer-lok syringes, one containing the aqueous phase, the other containing the oil and emulsifier, were cleared of air and linked by adapters and narrow gauge polyethylene tubing (Adams PE 190 or PE 200). The emulsion was made by repeatedly and forcefully ejecting the mixture from one syringe into the other through the narrow tubing. These preparations are generally referred to as Freund type adjuvants though variations are many (6).

Staphylococci.—Micrococcus pyogenes var. *aureus* (Stovall) was obtained from a stock culture maintained by Dr. R. J. Dubos. The properties of this culture have been outlined in a recent publication (10). For staphylococcal infections the culture was grown 18 hours in tryptone broth at 37°C. Each animal received 0.05 ml. of this culture in a total volume of 0.2 ml. by the intravenous route.

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[2] Although a systematic study of this variability has not been undertaken, certain factors in the purification of F I are thought to be important: (*a*) The composition of petroleum ether is variable at best, and the greater the amount of cyclic and aromatic hydrocarbons in the mixture, the greater the tendency for F I to be partially soluble. The use of purified single solvents has proved more satisfactory in this regard. (*b*) In the extraction and purification any contamination with water must be avoided. Both F I and F II have considerable affinity for water, sufficient, it is thought, to affect their behavior in organic solvents when but minute amounts of water are present. (*c*) Not surprisingly, it has been found that different germ mass preparations and different strains of tubercle bacilli (*i.e.* BCG) lead to variabilities.

Mice.—"Rockefeller Swiss" albino male mice were used in these studies. Received shortly after weaning, they were randomized into groups of 5, fed antibiotic-free pellets (Rockland mouse diet) and water *ad lib.* No terramycin was used in these experiments such as was the case in earlier studies (1).

Guinea pigs.—These animals were obtained through the generosity of Dr. Merrill Chase from a closed colony of pen-inbred albino guinea pigs of The Rockefeller Institute stock. They

TABLE II

Primary Toxicity of Methanol Extract Fractions by Intracutaneous Reactions in Tuberculin-Negative Guinea Pigs

Fraction	Guinea pig No.	Dose, mg. in 0.1 ml.	Reaction*	
			4 hrs.	24 hrs.
F I (6/19/58)	10-87	1.0	11 +	10 +
	10-88		10 +	12 ++
	10-87	0.5	9 ±	7 ±
	10-88		8 ±	6 ±
	10-87	0.1	7 −	0 −
	10-88		7 −	0 −
Serratia marcescens lipopolysaccharide	10-98		13 ++	16 ++±
	10-88	0.02	13 ++	14 ++
	12-98		12 +±	10 +
	10-87		12 +±	16 ++
	10-88	0.002	10 +	12 +±
	12-98		9 +	9 ±
	10-87		8 ±	8 ±
	10-88	0.0002	6 −	8 ±
	12-98		7 −	dot −

* The numbers refer to the diameter of the reaction site; − to +++ indicates intensity of the reaction. See Table I.

are free of Group C streptococci and were originally selected for high susceptibility to sensitization with 2:4 dinitrochlorobenzene.

EXPERIMENTAL

1. Several fractions from different preparations of methanol extract were compared for their ability to elicit an inflammatory response in guinea pigs at the site of an intracutaneous injection of 0.1 ml. volume containing the test materials in various concentrations. A lipopolysaccharide, the so called endotoxin, of *Serratia marcescens* was also used in the primary toxicity test so that the methanol extract fractions might be compared with a substance of established

potency. All sites were examined 4 hours following injection as well as the following day. In Table II are seen the responses to graded doses of F I (6/19/58) and *S. marcescens* lipopolysaccharide. F I (6/19/58) was slightly toxic (±) in 0.5 ml. doses and moderately toxic (+ to ++) in 1 mg. doses, but had still 500 times less potency than the endotoxin. Preparations F I (4/4/57) and F II

TABLE III

Secondary Toxicity: Weight Loss in Young Mice Induced by Intraperitoneal Injections of Killed Tubercle Bacilli and Methanol Extract Fractions

Treatment	Day weighed*					
		0	1	2	3	4
Normal, *not injected*	‡ $\bar{w}_n$	19.6	20.2	20.8	21.0	20.9
	Per cent $\Delta\bar{w}$	0	+3.0	+6.1	+7.1	+6.7
Water, *0.2 ml.*	$\bar{w}_n$ (*gm.*)	18.5	19.0	19.4	19.9	20.0
	Per cent $\Delta\bar{w}$	0	+2.5	+4.9	+7.6	+8.1
H37Rv, *5 mg.*	$\bar{w}_n$ (*gm.*)	18.8	17.1	18.2	18.6	18.7
	Per cent $\Delta\bar{w}$	0	−9.0	−3.2	−1.1	−0.5
ME, *5 mg.*	$\bar{w}_n$ (*gm.*)	18.6	18.0	19.0	19.2	19.1
	Per cent $\Delta\bar{w}$	0	−3.2	+2.2	+3.2	+2.7
F I-P, *5 mg.*	$\bar{w}_n$ (*gm.*)	18.2	17.4	18.2	18.6	18.7
	Per cent $\Delta\bar{w}$	0	−4.4	0	+2.2	+2.8
F IV, *5 mg.*	$\bar{w}_n$ (*gm.*)	19.6	17.5	18.9	19.5	19.7
	Per cent $\Delta\bar{w}$	0	−10.3	−3.6	−0.5	+0.5

* Day 0 is injection day; day 1 is day following injection.

‡ $\bar{w}_n$ = mean weight of 10 mice on day indicated; per cent $\Delta\bar{w} = 100\,\bar{w}_n/\bar{w}_o$ in which $\bar{w}_o$ is the mean weight on day of injection and $\Delta\bar{w}$ is the difference between $\bar{w}_o$ and $\bar{w}_n$ on day indicated.

(4/4/57), which might have been of this order of toxicity, were not tested in doses higher than 0.2 mg. The 24 hour reactions to the latter concentrations were negative in three animals, but in a later experiment F I (4/4/57) gave 8+ and 6± reactions in two tuberculin-negative guinea pigs (see Table VII). Preparation F IV (4/4/57) gave readings of 6+, 5±, and − in three different animals.

2. It being known that the intraperitoneal injection of dead mycobacterial bodies causes toxic reaction in mice, it was decided to find how ME, F I-P, and F IV would compare in this regard. A dose of 5 mg. was employed in each case. The mice were received on the day they were weaned and were held 8 days be-

fore treatment. Weights were recorded 7, 5, and 3 days before injection to ascertain that the animals were growing normally. The day of injection was considered day 0 of the experiment. The weight of the animals on that day, just prior to the injection, was that weight to which all subsequent weights were referred. Loss of weight and retarded recovery from loss of weight were considered indicators of secondary toxicity.

In Table III the mean gram weights ($\overline{w}$) for each group are recorded from day 0 up to 4 days post injection. Percentages of change (per cent Δ $\overline{w}$) from mean

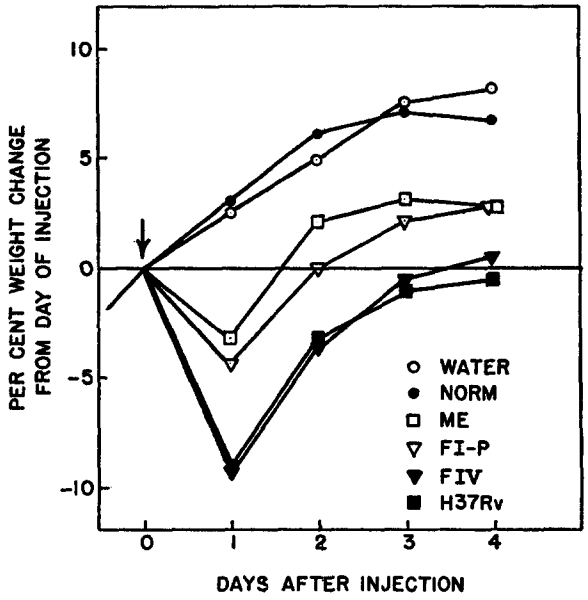

FIG. 1. Weight loss in young mice following intraperitoneal injection of large doses of killed H37Rv and methanol extract 8 days after weaning (↓). (○) normal weight gain curve, injection with 0.2 ml. water; (●) Normal weight gain curve, no injection; (□) 5 mg. methanol extract; (▽) 5 mg. F I-P; (▼) 5 mg. F IV; (■) weight change curve for mice injected with 5 mg. killed H37Rv.

weight at day 0 ($\overline{w}_0$) at each subsequent weighing are also included in the table. Fig. 1 is the plot of these percentages. It is clearly seen that all materials in the doses employed induce an immediate absolute loss of weight and not merely a decrease in rate of weight gain. Presumably a dose level could be found for all of these substances which would eliminate weight gain for a 24 hour period without causing an actual loss. ME and F I-P entrain the least loss in the 5 mg. dose; and recovery of initial weight ($\overline{w}_0$) occurs within 2 days, per cent Δ $\overline{w}$ passing from a negative to a positive quantity. Killed H37Rv and F

IV induce the greatest loss, and recovery from their effect requires 4 days from the time of injection. It is apparent from the shape of the weight change curves (Fig. 1) that it would take many days for the affected mice to attain the high positive $\Delta \overline{w}$ achieved by the normal or the water-injected groups 4 days post injection. No effect on the weight gain curve was observed when mice were injected with 1 mg. of F I-P or with 1 mg. of F IV.

This loss of weight may be simply a result of interruption of food and water intake. That this is the case is suggested by a control experiment in which killed H37Rv was injected into two groups of mice. Food and water were withheld from one group for 24 hours, during which time the injection-induced weight loss occurs. Another group was injected with water and similarly starved for 24 hours. All three groups lost approximately the same percentage of their pre-injection weight. The non-starved, water-injected controls were unaffected.

The physiological or chemical effect of these substances that result in voluntary restriction of food and/or water intake is unknown. It has been reported that endotoxins, which in considerably smaller doses may function in the same manner, inhibit the motility of the gastrointestinal tract and block the absorption of food (11). Preliminary experiments which will be described at a later date seem to indicate that the toxic effect induced by F I-P, but not by whole killed H37Rv, depends on prior exposure to or presence of substances of microbial or parasitic origin. This appears to be equally true for many of the observed biological effects of endotoxins from the Gram-negative bacteria.

3. In an experiment not reported in detail, F I (4/4/57) and F II (4/4/57) were tested for their ability to depress resistance of mice to staphylococcus infections. Solutions of these substances were mixed with the suspension of staphylococcus in such a way that each mouse received 2 mg. intravenously with the challenge dose of bacteria. As routine this dose was 0.05 ml. of an 18 hour culture diluted fourfold with saline for injection.

It was found that both F I and F II depressed resistance to such an extent that the *median*, *mean*, and *mean cumulative average*[1] survival times were shortened by at least half. Schaedler and Dubos (2) found 0.1 mg. of heat-killed BCG cells to be effective when used in this way.

Subfractions of F I (4/4/57) were tested in the same manner. F I-P was administered in 2.0 and 0.5 mg. doses to two groups of ten mice. Although the doses of F I-S employed were approximately a tenth of these, F I-S comprises but a tenth at most of the total fraction F I; and 0.05 mg. of F I-S has been shown to be effective in increasing resistance of mice to tuberculosis (1). The survival times are recorded in Table IV A and their cumulative averages in Table IV B.[1] Plots of the cumulative average survival times in Fig. 2 demonstrate graphically the difference between the treatment with the F I-S subfraction and with F I-P. In spite of the disparity in dosage, it may be asserted that F I-S is considerably less toxic than F I-P. Of the latter 0.5 mg. was suffi-

cient to effect a three-to fourfold decrease of the *mean cumulative average* survival time, while 0.2 mg. of F I-S had no more effect than saline.

TABLE IV A

Depression of Resistance to Staphylococcus Infection by Simultaneous Administration of FI Subfraction
Individual Survival Time, Days

Order of death	FI subfraction added to staphylococcus challenge dose				
	Saline	F I-P 2.0 mg.	F I-P 0.5 mg.	F I-S ~0.2 mg.	F I-S ~0.05 mg.
1	5	1	1	4	4
2	5	1	1	5	5
3	5	1	1	6	6
4	5	1	2	7	7
5	8	1	2	7	7
6	8	1	3	8	7
7	8	2	5	9	8
8	8	2	6	S	8
9	9	4	8	S	S
10	—	5	—	—	S

TABLE IV B

Depression of Resistance to Staphylococcus
Cumulative Average Survival Time

Order of death	FI subfraction added to staphylococcus challenge dose				
	Saline	F I-P 2.0 mg.	F I-P 0.5 mg.	F I-S ~0.2 mg.	F I-S ~.05 mg.
1	5	1	1	4	4
2	5	1	1	4.5	4.5
3	5	1	1	5	5
4	5	1	1.25	5.5	5.5
5	5.6	1	1.4	5.8	5.8
6	6	1	1.7	6.2	6
7	6	1.1	2.2	6.6	6
8	6.2	1.2	2.6	S	6.2
9	6.6	1.6	3.2	S	S
10	—	1.9	—	—	S
MCA*	5.6	1.0	1.6	6.0	5.8

* Mean cumulative average (see footnote 1).

4. Lipopolysaccharides from Gram-negative bacteria also depress resistance to infections if administered at or shortly before the time of challenge (2, 12, 13). Another property of the lipopolysaccharides is their ability to combine

with properdin (5, 14, 15). F I, F I-S, and F I-P were examined by Dr. Maurice Landy for their ability to neutralize properdin in fresh human serum. The test system was an adaptation of the coliphage assay for properdin, in which the test materials which interact with properdin block the inactivation of TR2+ phage by fresh human serum (5, 15). It was found that both the F I parent fraction and the F I-P subfraction were highly active and more or less comparable. They appeared to inhibit phage inactivation as strongly as the most active lipopolysaccharides. F I-S on the other hand was determined to be of very low potency in this test showing at best one-tenth the activity of F I-P on a weight-for-weight basis.

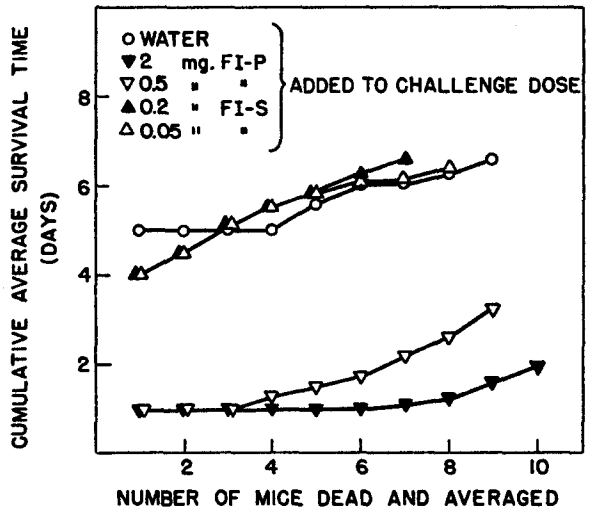

FIG. 2. Depression of cumulative average survival time (see footnote 1) of mice infected with staphylococcus. Subfractions of methanol extract were administered intravenously with the challenge. (○) water added to challenge; (▼) mice received 2.0 mg. F I-P; (▽) mice received 0.5 mg. F I-P; (▲) mice received approximately 0.2 mg. F I-S; (△) mice received approximately 0.05 mg. F I-S.

5. The results of an experiment testing the skin reactive antigen content of F I, F II, F IV, and AEP, all from ME (4/4/57), are compiled in Table V.

An investigation was made of the content of skin-reactive antigens in animals that had been sensitized with mycobacterial cells and were consequently sensitive to intradermal injection of tuberculin (PPD). In the first experiment (Table V), guinea pigs were used that had been given an intramuscular vaccination with living BCG 6 weeks before skin testing. These animals were moderately sensitive to PPD in 5 μg. doses, as shown. The conclusion to be drawn from this first experiment is that F IV in 50 μg. dose and AEP in 5 μg. dose may contain tuberculin type antigens or other materials that give direct, although not

TABLE V

Tuberculin Sensitivity of Guinea Pigs Sensitized with Living BCG. Tuberculin Type Activity of Methanol Extract Fractions in Tuberculin-Sensitive Guinea Pigs

Test antigen	Dose	Guinea pig No.	Reaction	
			24 hrs.	48 hrs.
	μg.			
PPD	5	33–05	12 ++	13 +++
		31–22	9 +	10 ++
		32–85	10 ++	10 ++
		32–86	12 ++	14 +++
		32–47	10 ++	10 ++
		32–38	9 +	9 +
F I (4/4/57)	50*	33–05	0 −	0 −
		31–22	6 +	7 +
		32–85	0 −	0 −
		32–86	0 −	0 −
		32–47	0 −	0 −
		32–38	0 −	0 −
F II (4/4/57)	50*	33–05	0 −	0 −
		31–22	5 ±	6 ±
		32–85	0 −	0 −
		32–86	0 −	0 −
		32–47	Spot −	Spot −
		32–38	0 −	0 −
PPD	5	33–36	10 ++	11 ++
		33–37	10 ++	11 ++
		33–73	14 +++	13 +++
		33–14	12 ++	14 +++
		33–19	10 ++	10 ++
F IV (4/4/57)	50‡	33–36	8 +	10 ++
		33–37	6 ±	8 +
		33–73	6 ±	8 +
		33–14	5 ±	6 ±
		33–19	9 +	12 ++
AEP	5§	33–36	10 ++	10 ++
		33–37	6 ±	8 +
		33–73	5 ±	5 ±
		33–14	Spot −	6 ±
		33–19	Spot −	Spot −

* 200 μg. doses as toxicity controls in seven non-sensitized animals gave no reaction at 24 hours.

‡ 200 μg. doses in seven non-sensitized animals gave five negative, one 6 +, and one 5 ± reactions at 24 hours.

§ 5 μg. doses in seven non-sensitized animals gave one 5 ± and six negative reactions at 24 hours.

TABLE VI

Tuberculin Sensitivity of Guinea Pigs Sensitized with Killed Tubercle Bacilli in Paraffin Oil and Vaseline. Tuberculin Type Activity of Methanol Extract Fractions

Test antigen	Dose	Guinea pig No.	Reaction	
			24 hrs.	48 hrs.
PPD	1 μg.	10–57*	14 +++	13 +++
		10–58	12 +++	13 ++
		10–59	13 ++	11 +
		10–60	14 ++	15 +++
F I (6/19/58)	100 μg.	10–57*	10 +	9 +
		10–58	8 +	–
		10–59	10 +	–
		10–60	11 +	11 +
	Toxicity controls, 100 μg.	10–85‡	7 ±	–
		10–86	5 +	–
F I (4/4/57)	200 μg.	40–08§	10 +	8 ±
		40–12	8 +	9 +
	Toxicity controls, 200 μg.	49–01‡	8 +	5 –
		49–02	6 ±	–
F I (2/18/58)	50 μg.	11–93‖	13 ++	8 +
		11–98	14 +++	11 ++
		12–00	13 +++	8 +
		12–01	12 ++	13 +++
	Toxicity controls, 50 μg.	49–64‡	–	–
		49–65	5 –	–
F II (2/18/58)	50 μg.	11–93‖	–	–
		11–98	–	–
		12–00	–	–
		12–01	–	–
	Toxicity controls 50 μg.	49–64‡	–	–
		49–65	–	–

* 80 gm. tubercle bacilli in oil and vaseline 5 weeks before test.

‡ Non-sensitized controls for primary toxicity.

§ 500 gm. tubercle bacilli in oil and vaseline 10 weeks before test.

‖ 200 gm. tubercle bacilli in oil and aquaphor 10 weeks before test.

strong, reaction on animals that have been sensitized by this method. The results with F I and F II provided no information of significance.

The next experiments were conducted in guinea pigs which had been rendered much more highly sensitive to tuberculin by injection with killed tubercle bacilli in paraffin oil and vaseline. While the modes of sensitization of the animals used (10 in number) were various, all still retained a high degree of reactivity to PPD, *e.g.* animals 10-57 to 10-60 reacted to 1 μg. of PPD with a 13++ to a 14+++ at 24 hours, and the reactions persisted intense at 48 hours. From these more crucial tests, it can be seen (Table VI) that F II in 50 μg. doses again appears to be devoid of tuberculin type reactivity, but F I is now seen definitely to produce reactions, in contrast to the data of Table V secured in animals possessing a weaker sensitization. The F I (2/18/58) preparation is definitely laden with substances having, apparently, tuberculin-like reactivity. The other F I preparations (6/19/58 and 4/4/57) react definitely but in relation to their dose (100 to 200 μg.) only moderately.

6. Five guinea pigs (Nos. 49-05, 49-07, 49-15, 26-46, 26-58) received a Freund type emulsion of F I (4/4/57) in the neck muscles. The total injection volume per animal was 1 ml. divided equally among five sites. The emulsion contained 200 μg. killed BCG and 200 μg. F I per ml. After 5 weeks the animals received intracutaneous injection of 200 μg. F I in 0.1 ml.

Two guinea pigs which had been sensitized with tubercle bacilli were used to control the ability of complete adjuvant alone to induce sensitivity to skin reactive antigens in F I. Three guinea pigs which had received F I alone in a much larger dose had already been skin-tested with F I and were included in the table. Primary toxicity of F I was controlled in animals which had received the water-in-oil emulsion containing neither F I nor tubercle bacilli.

The 24 hour and 48 hour reactions are recorded in Table VII. Immediately apparent are that F I (4/4/57) in 200 μg. doses is mildly toxic; that F I in complete Freund type adjuvant does elicit sensitivity to F I components significantly more intense than does tubercle bacilli alone; but that F I without tubercle bacilli as adjuvant will not function as a sensitizing antigen, at least not by this technique.

F I-S and F I-P were prepared from F I (4/4/57) and used to skin test F I-sensitive guinea pigs 26-58 and 49-07. Toxicity was controlled as previously in two animals (Nos. 34-97 and 49-01) which had been injected with an emulsion containing only picrylated homologous red blood cell stromata. Tuberculin activity was also controlled in two animals (Nos. 40-13 and 40-14). Graded doses of 200, 67, and 22 μg. of F I-P and estimated 67 and 22 μg. doses of F I-S were administered to all animals. Concentrations of F I-S solutions are difficult to determine by dry weight owing to the relatively large amounts of salt introduced during the preparation of F I-S. The doses of F I-S are therefore approximations based on the assumption that F I-S comprises one-tenth of the total F I.

The reactions recorded in Table VIII demonstrate that F I specific sensitizing antigen may be found in the F I-P subfraction, but not in F I-S.

7. An attempt was made to find whether any ME fraction contained substances that would duplicate the known adjuvant effect of dead mycobacterial bodies. For this purpose, F I, F II, F IV, or AEP was substituted for the myco-

TABLE VII

Sensitizing Capacity of F I (4/4/57) in Freund Type Adjuvant
Intracutaneous Reactions Elicited by F I

Preparation of guinea pigs	Test dose Test date	Guinea pig No.	Reaction	
			24 hrs.	48 hrs.
F I + tubercle bacilli 200 μg. F I (4/4/57) 200 μg. BCG, intramuscular injection in water-in-oil emulsion, Dec. 18, 1957	200 μg. Jan. 21, 1958	49–05	15 +++	14 +++
		49–07	12 ++	13 +++
		49–15	12 ++	11 ++
		26–46	11 +	5 −
		26–58	12 ++	13 ++
Tubercle bacilli (no F I) 500 μg. tubercle bacilli (Jamaica No. 22), intramuscular injection in oil and vaseline Nov. 6, 1957	200 μg. Jan. 21, 1958	40–08	10 +	8 ±
		40–12	8 +	9 +
F I (no tubercle bacilli) 750 μg. F I (4/4/57), intramuscular injection in water-in-oil emulsion, Oct. 29, 1957	100 μg. Nov. 26, 1957	14–69	8 +	<5 ±
		14–84	8 +	<5 ±
		24–64	8 +	5 +
Toxicity control (no F I, no tubercle bacilli) Intramuscular injection, water-in-oil emulsion containing picrylated red blood cell stromata, Dec. 18, 1957	200 μg. Jan. 21, 1958	49–01	8 +	5 −
		49–02	6 ±	Dot −

bacteria in a method for sensitizing guinea pigs to picryl chloride (contact skin reactivity). Picrylated homologous erythrocyte stromata were included into a water-in-oil emulsion containing adjuvant and injected into guinea pigs by the intramuscular route according to Chase (8). Unfortunately it was discovered that the animals in question had been sensitized previously with 2,4-dinitrochlorobenzene, sensitivity to which shows a weak degree of cross-reactivity with the chemically related trinitrochlorobenzene (picryl chloride).

When the dinitrochlorobenzene stock employed was actually tested with picryl chloride, only a few, spotty reactions were observed. Animals which had received F I and F II as adjuvant substitutes showed no differences from

TABLE VIII

Distribution of F I-Sensitizing Antigen in Subfractions F I-P and F I-S of F I (4/4/57)

Preparation of guinea pigs	Guinea pig No.	Test antigen	Dose	Reactions	
				24 hrs.	48 hrs.
			μg.		
F I + tubercle bacilli 200 μg. F I, 200 μg. BCG in oil-aquaphor emulsion; intramuscular, Dec. 18, 1957	26–58	F I-P	200	16 +++	15 +++
			67	14 +++	12 ++
			22	11 ++	11 ++
		F I-S	~67	6 −	<5 −
			~22	5 −	Dot −
	49–07	F I-P	200	13 +++	13 +++
			67	11 ++	10 +
			22	5 −	Dot −
		F I-S	~67	0 −	<5 −
			~22	0 −	Dot −
Tubercle bacilli (no F I) 500 μg. killed tubercle bacilli (Jamaica No. 22) in oil-vaseline; intramuscular, Nov. 6, 1957	40–13	F I-P	200	7 +	6 ±
			67	6 −	6 ±
			22	6 −	5 −
		F I-S	~67	4 −	Spot −
			~22	? −	Dot −
	40–14	F I-P	200	12 ++	10 +
			67	10 +	8 ±
			22	6 −	6 −
		F I-S	~67	4 −	Dot −
			~22	0 −	Dot −
Toxicity control (no F I, no tubercle bacilli) Oil-aquaphor emulsion of picrylated homologous red blood cell stromata; intramuscular, Dec. 18, 1957	34–97	F I-P	200	Spot −	<5 −
			67	Dot −	Dot −
			22	0 −	0 −
		F I-S	~67	0 −	0 −
			~22	Dot −	0 −
	49–01	F I-P	200	6 −	Dot −
			67	0 −	0 −
			22	0 −	0 −
		F I-S	~67	0 −	0 −
			~22	0 −	0 −

these controls. The replacement of mycobacteria by F IV or AEP, on the other hand, was accompanied by a mild but definitely increased hypersensitivity to picryl chloride in most of the animals thus treated.

However, since 0.8 mg. of the various fractions were substituted for mycobacteria, an amount at least tenfold larger than required of mycobacteria for the induction of hypersensitivity, it may be assumed that if any of the fractions of methanol extract or acetone extract had adjuvant activity, it was very weak. F I and F II may be considered devoid of adjuvant activity in this system. F IV and AEP or some component of these does have adjuvant activity; but in view of the large amounts used it is not impossible that that component could be bacillary fragments.

CONCLUSIONS

Although subfraction F I-S of methanol extract and mixtures of fraction F I with fraction F II have been shown to have promising prophylactic activity against experimental tuberculosis in mice (1), the ideal substance should be free of toxic and allergenic properties. From the experiments presented in this study, the following conclusions can be drawn:

1. F I in moderate doses can be mildly toxic to the skin; although as much as 1 mg. of certain preparations can be injected with slight, if any, irritation.
2. F I and F II are both capable of temporarily depressing resistance to certain bacterial infections. In the case of F I this activity seems confined to the F I-P subfraction.
3. F I-P in large doses is also capable of causing marked weight loss in growing mice.
4. Properdin is readily neutralized by F I-P, indeed as readily as by lipopolysaccharides of Gram-negative bacteria. F I-S does not possess this ability.
5. Certain F I preparations contain antigens which elicit an allergic skin reaction in guinea pigs sensitized with killed tubercle bacilli. This may be a purification problem since the property seems to vary among the preparations tested.
6. A component of F I is capable of sensitizing guinea pigs by a single injection in a water-in-oil emulsion containing whole killed tubercle bacilli. The sensitivity is distinct or considerably more intense than that elicited by tubercle bacilli alone, but is dependent on the presence of tubercle bacilli as adjuvant. This antigen is characteristic of F I-P but appears absent from F I-S.
7. Neither F I nor F II used as adjuvant is capable of inducing sensitivity to picryl chloride in guinea pigs. It was previously thought that one of these might possess adjuvant activity since their admixture was a far more effective vaccine against experimental tuberculosis than was either alone in even larger doses. The assumption made had been that F II was an adjuvant for the activity of F I and its protective effect. If this is true, however, such adjuvant activ-

ity is apparently not of general applicability but is perhaps confined to mixtures with F I or other substances increasing resistance to bacterial infections by the same mechanisms.

8. The results of the several experiments reported here have served to demonstrate that subfraction F I-S from methanol extract possesses slight, if any, toxic or allergenic properties by the criteria imposed. It is suggested, therefore, that this substance qualify as a suitable non-living vaccine against tuberculosis.

SUMMARY

Fractions of methanol extract which had been previously demonstrated to increase the resistance of mice to experimental tuberculosis have been subjected to an examination of their toxic and allergenic properties.

The criteria for toxicity were: (*a*) production of inflammatory skin reactions in guinea pigs; (*b*) induction of weight loss in mice by intraperitoneal injection; and (*c*) depression of resistance to staphylococcus infections in mice.

Allergenicity of a preparation was investigated by (*a*) its ability to evoke a hypersensitive skin response in guinea pigs previously sensitized with whole tubercle bacilli; and (*b*) its capacity to induce hypersensitivity to one or more of its components when injected under appropriate conditions into guinea pigs.

Fraction F I, a preparation precipitated from methanol extract by slow concentration at 45°C., was found to possess some toxicity and some allergenicity by all of the criteria employed.

Subfraction F I-P, precipitated from aqueous suspensions of F I by 33 per cent ethanol and 0.5 per cent NaCl, was apparently the F I component responsible for these activities. The saline-ethanol-soluble subfraction, F I-S, was neither toxic nor allergenic by the tests performed.

These findings were considered of particular interest inasmuch as F I-S, despite its small yield, had been shown earlier to be the most active single substance used as vaccine to increase resistance to experimental tuberculosis in mice.

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