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Comparative Study of *in vivo* and *in vitro* Grown *Mycobacterium tuberculosis*. IV. Immunogenic Differentiation.* (29801)

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In a comparative study of the biochemistry, pathogenicity and immunogenicity of *in vivo* and *in vitro* grown *Mycobacterium tuberculosis*, strain H37Rv, significant differences were found to exist(1,2). Whereas the *in vivo* grown bacillus exhibited double the virulence for mice as measured by mean survival time, its immunizing capacity using phenol-killed vaccines was lower. This latter observation was true regardless of whether the immunized mice were challenged with tubercle bacilli grown *in vivo* or *in vitro*. The following report involves an attempt to extend this finding of differential immunogenicity of *in vivo* and *in vitro* cultured *M. tuberculosis*, and thereby to gain some insight into the mechanism involved.

Methods. The strain of *in vitro* tubercle bacilli used in this study was H37Rv, grown on the surface of Kirchner's synthetic liquid medium. The growth of the *in vivo* bacilli was carried out in mice (strain CF1) which were initially infected with strain H37Rv grown *in vitro*, but thereafter by successive transfers from mouse to mouse with the *in vivo* grown bacilli. The separation by differential centrifugation of the *in vivo* grown tubercle bacilli (LRv†) from tuberculous mouse lung homogenates was carried out according to the procedure previously described (1). It has been found that there is no significant difference, with regard to biochemical, pathogenic and immunogenic characteristics, in the LRv separated after one mouse passage and the LRv separated after 25 serial mouse passages.

Degree of immunity was determined by recording individual survival times of vaccinated mice (strain CF1, 6 weeks old, female) after challenge infection, with confirmation by necropsy that the cause of death was tuberculosis. The mice were divided into 8 vaccinated groups of 15 mice each, in addition to control groups of non-vaccinated mice which were injected with phosphate buffer or adjuvant. Each group was vaccinated twice, at 1-week intervals, subcutaneously with 0.1 ml volumes of various vaccine preparations. Four groups received different vac-

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† LRv is the designation used throughout for the *in vivo* grown tubercle bacilli, separated by differential centrifugation from lung homogenates of tuberculous mice initially infected with *in vitro* grown strain H37Rv, and maintained thereafter by successive transfers from mouse to mouse.

cine preparations of LRv, and 4 groups received corresponding vaccine preparations of H37Rv. The vaccine preparations used were: 0.5 mg dry weight amounts of LRv and H37Rv, suspended in phosphate buffer pH 7.4 and heated at 70°C for 30 minutes; the same heat-killed preparations but made up in water-in-oil emulsion using Difco's Freund Incomplete Adjuvant; 0.01 mg dry weights of whole viable cells suspended in phosphate buffer pH 7.4; and 0.1 mg dry weights of unfractionated sonicates, 99% disrupted with 37 μ glass beads for 20 minutes in a Raytheon 9KC Magnetostriction Oscillator model S-102A. Challenge infection was performed one week after the second vaccination by intravenous injection of 0.2 ml volume containing 0.05 mg dry weight of LRv, separated on the same day from tuberculous lung homogenates.

Comparative elicitation of a delayed hypersensitive response was determined by measuring the diameter and thickness of induration, and observing the degree of erythema and central necrosis at 3, 24, and 48 hours after intradermal injection of 0.1 ml of provoking materials into tuberculous guinea pigs. Random bred albino guinea pigs were employed, and were infected with *in vitro* grown strain H37Rv by the airborne route 6 weeks previous to skin testing (through the courtesy of Dr. M. L. Cohn at National Jewish Hospital in Denver). Provoking materials were as follows: Old Tuberculin (OT); buffered suspensions of H37Rv and LRv, heat treated at 95°C for 1 hour, in the amount of 0.005 mg dry weight per injection dose.

Comparative induction of delayed hypersensitivity was determined using New Zealand White rabbits which were injected subcutaneously with 1.3 mg amounts of heated (95°C for 1 hour) H37Rv or LRv in adjuvant 3 months prior to skin testing, with 3 boosters in the interim. Provoking materials were the same used in the elicitation experiment, except for a 10-fold increase in the dose of H37Rv and LRv.

Results and discussion. Immunizing activities. The results graphed in Fig. 1 and 2 demonstrate that various vaccine preparations of LRv consistently exhibited a lower immunizing capacity against acute tuberculous infection in mice, in comparison to the corresponding preparations of H37Rv. These findings corroborate the previously reported result for phenolized vaccines(2). Values shown in Fig. 1 and 2 are derived from the results of a representative experiment, in which the logarithms of the per cent survivors, at each point where one or more deaths occurred, are plotted against days of

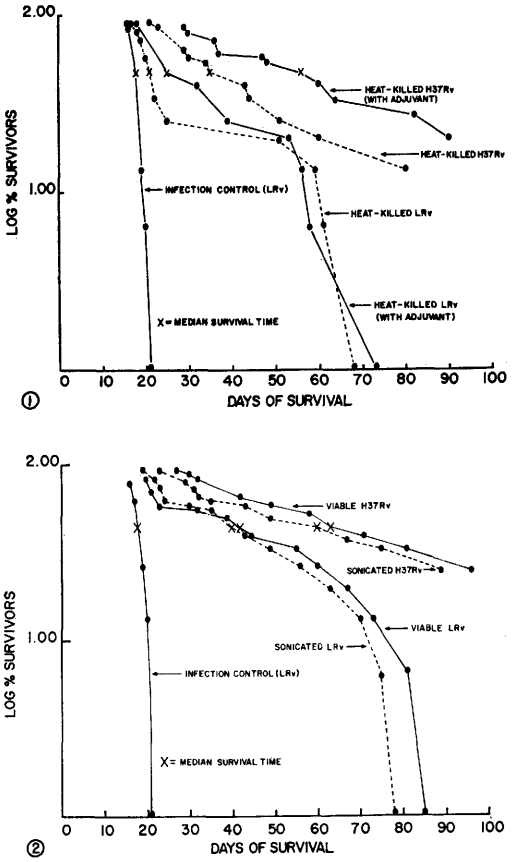

FIG. 1. Comparative degrees of immunity conferred on mice by heat-killed vaccines, with and without adjuvant, of *in vivo* (LRv) and *in vitro* (H37Rv) grown *M. tuberculosis*. For heat-killed vaccines without adjuvant, $P < 0.05$; for heat-killed vaccines containing adjuvant, $P < 0.01$.

FIG. 2. Comparative degrees of immunity conferred on mice by viable and sonicated vaccines of LRv and H37Rv. For viable vaccines, $P < 0.05$; for sonicated vaccines, $P < 0.01$. LRv is the designation used for *in vivo* grown tubercle bacilli, separated by differential centrifugation from lung homogenates of mice initially infected with *in vitro* grown strain H37Rv, and maintained thereafter by successive transfers from mouse to mouse.

survival at that point. Statistical significance of the differences between the LRv and H37Rv vaccines was established by $P<0.05$ for heated and viable vaccines, and $P<0.01$ for sonicated and heated-adjuvant vaccines. This experiment was repeated 4 times with similar results. The significance of these results of differential immunizing capacities of LRv and H37Rv is further illustrated by the following indices: differences in median survival times (as indicated by X's in Fig. 1 and 2); differences in slopes of the curves of Log % Survivors; differences in numbers of mice surviving any period of infection, particularly the percent remaining alive at termination of the experiment; and the consistency of the effect for all vaccine preparations in replicate experiments.

It would thus appear that the deficient immunogenicity of LRv in comparison to that of H37Rv is a generalized phenomenon, regardless of mode of vaccine preparation. This phenomenon may seem paradoxical in view of the fact that LRv has twice the virulence of H37Rv. However there is precedent for a negative correlation of virulence and immunogenicity of strains used for vaccination in other diseases as well as for tuberculosis, where for example, viable vaccines of non-infectious, avirulent strain H37Ra were shown to be equal or superior to viable vaccines of mildly-infectious, attenuated strain BCG(3). Consistent with this latter finding is the preliminary evidence that there is a greater degree of multiplication of LRv in mice than there is of H37Rv. In turn it is feasible to postulate that the deficient immunizing capacity of LRv is the cause of its increased virulence, taking into account the prolonged, chronic type of infection that is characteristic of tuberculosis, where immunity factors are significant in determination of virulence.

One possible explanation for these results might be that LRv is deficient in constituents with adjuvant activity, in view of its lower chloroform soluble lipid(4). Against this hypothesis is the finding shown in Fig. 1 that adjuvant did not raise the level of immunization of LRv up to that of H37Rv, with or without adjuvant. It may be of further significance that adjuvant has a lesser enhancing effect on LRv vaccines than on vaccines of H37Rv. The operation of several other possible variables in producing the effect of deficient immunogenicity of LRv is discounted by the results presented in Fig. 1 and 2, namely, the manner of killing cells (phenol or heat gave the same result); the effect of killing *per se* (viable cells of LRv also produced decreased protection); a masking effect of surface or cell wall antigens (shown by Larson *et al*(5) to possess major tuberculo-immunogenic activity) by the unusual hydrophilic surface property of LRv (sonically disrupted cells of LRv, unfractionated so as to include all constituents, also exhibited lower immunogenicity than H37Rv).

Several other hypotheses to explain the deficient immunogenicity of LRv are suggested. 1) The *in vivo* grown bacillus is truly deficient in immunogenic components. This explanation is supported by the finding that it is low in its content of chloroform soluble lipid(4), which aside from containing constituents with adjuvant activity (Wax D) also contains Wax B, which has been reported to be the key lipid fraction responsible for tuberculo-immunity(6). 2) *In vivo* bacilli, being separated from the lungs of mice which are in the terminal stages of tuberculous infection, have specific circulating antibody adsorbed to their surface, which results in decreased immunogenicity. There is some precedent for such an effect in the recent report by Crowle and Hu(7). A related hypothesis would be that non-specific inhibitors of immunity are adsorbed by LRv from the lung environment. 3) LRv loses antigenic components, operative in tuberculo-immunity, in the process of its mechanical separation from lung tissue in an isotonic sucrose medium. These various hypotheses are being tested.

Elicitation and induction of delayed hypersensitivity. Results of a comparison of the abilities of LRv and H37Rv to elicit and induce delayed hypersensitivity are presented in Tables I and II. The conditions of the experiments are described in the footnotes of the Tables. Consistent with the results for

TABLE I. Comparative Elicitation by *in vivo* and *in vitro* Grown *M. tuberculosis* of Delayed Hypersensitive Response in Tuberculous Guinea Pigs.*

Provoking material†	Responses‡ 48 hr after injection of provoking material: Induration (mm)	Thickness (mm)	Erythema	Central necrosis
1/20 Dil. OT	18	4	+	+
Heated H37Rv	19	4	+	+
" LRv§	8	1	+	—

* Guinea pigs were infected with *in vitro* grown strain H37Rv by the airborne route 6 wk previously.

† Intradermal injection of 0.1 ml of OT (Old Tuberculin); or of H37Rv or LRv suspension (0.005 mg dry wt), heat treated at 95°C for 1 hr.

‡ Readings taken at 3, 24, 48 hr. Results recorded above are 48 hr readings. Values for induration and thickness are each the mean of 4 observations (2 per guinea pig, 2 guinea pigs per group). Non-sensitized normal control guinea pigs were negative when separately injected with the 3 provoking materials.

§ LRv is the designation used for *in vivo* grown tubercle bacilli, separated by differential centrifugation from lung homogenates of mice initially infected with *in vitro* grown strain H37Rv, and maintained thereafter by successive transfers from mouse to mouse.

immunizing capacity, the *in vivo* bacillus exhibits a lesser potential for both eliciting and inducing delayed hypersensitivity. This is shown in Table I by the less than one-half the induration and the lack of central necrosis produced in the skin of tuberculous guinea pigs when LRv is used as provoking material. This experiment was preliminary to the more complete experiment summarized in Table II. Again LRv used as provoking material in skin reactions consistently gave a lowered response. Old Tuberculin used in varying concentrations as provoking material clearly indicated that rabbits previously sensitized with LRv exhibited a deficient state of delayed hypersensitivity in comparison to rabbits sensitized with H37Rv. Finally, in the most convincing test, rabbits previously sensitized with LRv and then skin tested with LRv showed no delayed hypersensitive response in concentrations of LRv employed. The very same conditions in rabbits where H37Rv was substituted for LRv produced a typical tuberculin skin reaction.

Larson *et al*(5) have discussed the precedents for differentiation of delayed hypersensitivity reactions induced and elicited in experimental animals by different species and strains of mycobacteria. A similar phenomenon may be involved in the observed sensitizing differences between LRv and H37Rv, although evidence to date indicates that these are not 2 genotypically different strains but rather phenotypically adapted variants. The correlation of LRv's decreased ability to protect mice against tuberculosis and its deficient sensitizing capacity may be significant. It is consistent with the usually, but not necessarily, observed tuberculin allergy accompanying tuberculo-immunity(8). The 3 hypotheses formulated previously in this paper to account for the lower immunizing capacity of LRv apply equally well to explain the observed deficiency in elicitation and induction of the delayed hypersensitive state.

Summary. A comparative study of the immunogenic properties of *in vivo* and *in vitro* grown *M. tuberculosis* of the same strain H37Rv has corroborated and reinforced the hypothesis that a wide biological range of significant differences exists between the two types. The *in vivo* grown organism, regardless of mode of vaccine preparation, consist-

TABLE II. Comparative Induction of Delayed Hypersensitivity in Rabbits by *in vivo* and *in vitro* Grown *M. tuberculosis.**

Provoking material†	Responses‡ 48 hr after injection of provoking material: Sensitized with H37Rv: Induration (mm)	Central necrosis	Sensitized with LRv: Induration (mm)	Central necrosis
Conc OT	26	+	16	—
1/2 Dil. OT	18	+	9	—
1/5 " "	10	—	4	—
1/10 " "	6	—	2	—
Heated H37Rv	18	+	8	—
" LRv	6	—	—	—

* Rabbits were injected subcutaneously with 1.3 mg amounts of heated LRv or H37Rv in adjuvant 3 mo prior to skin testing, with 3 boosters in the interim.

† Intradermal injection of 0.1 ml of varied concentrations of OT (Old Tuberculin); or of H37Rv or LRv suspensions (0.05 mg dry wt), heat treated at 95°C for 1 hr.

‡ Readings taken at 3, 24 and 48 hr. Results recorded above are the 48 hr readings. Values for induration are each the mean of 8 observations (2 per rabbit, 4 rabbits per group). Non-sensitized normal control rabbits were negative when separately injected with the 6 provoking materials.

ently exhibited a deficient immunizing capacity against acute tuberculous infection in mice, in comparison to the corresponding preparations of the *in vitro* grown organism. Furthermore, the *in vivo* grown bacillus exhibits a lesser potential for both inducing and eliciting the delayed hypersensitive state in experimental animals. Several hypotheses are suggested to account for these differences.

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Endotoxin Detoxification by Guinea Pig Tissue Homogenates and Possible Significance of This Reaction *in vivo*. (29802)

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Homogenates of various animal tissues are capable of enzymatically detoxifying bacterial endotoxins *in vitro*. Trapani *et al*(1) found endotoxin-detoxifying activity in rabbit liver, adrenal, kidney and lymph node but not in brain, heart or spleen. Keene(2), on the other hand, detected activity in rabbit spleen and heart, as well as in liver and kidney. Smith *et al*(3) found dog spleen to be more active than liver, whereas, kidney, heart and skeletal muscle were inactive. Corwin and Farrar(4) investigated the mechanism by which endotoxin is inactivated by guinea pig liver mitochondria, and concluded that inactivation is accomplished by a process of activation and subsequent oxidation of the fatty acid portion of the endotoxin molecule.

None of these studies provides information about the possible physiological significance of enzymatic detoxification by tissues in the response of normal animals to endotoxin administration. Formal *et al*(5) found that guinea pigs with hepatic necrosis due to carbon tetrachloride (CCl_4) were abnormally susceptible to the lethal action of endotoxin. Farrar and Magnani(6) showed that the degree of susceptibility parallels the severity of the liver damage, and that the response of the animal returns toward normal as the hepatic lesion heals. Furthermore, damaged liver tissue showed almost no ability to detoxify endotoxin *in vitro*, whereas normal liver was quite active. Liver tissue removed after regeneration had taken place showed near normal activity. They suggested that CCl_4 may induce abnormal susceptibility to endotoxin by interfering with the ability of the liver to detoxify this toxic bacterial product.

Although the liver appears to be the principal site of removal of endotoxin from the circulation(7), the spleen also participates under certain circumstances. Wiznitzer *et al* (8) have shown that the dog spleen is capable of both removing and detoxifying endotoxin *in vivo*, whereas the kidney is not. While other organs may possess the necessary enzymes for inactivating endotoxin, only the liver and spleen appear to have access to sufficient amounts of injected endotoxin to function as important sites of endotoxin degradation.

This report describes the *in vitro* inactivation of endotoxin by various tissues of the guinea pig, and attempts to assess the physiological role of the spleen in the response of normal animals and those with liver damage to administration of endotoxin.