

IMMUNIZING CONSTITUENTS OF THE TUBERCLE BACILLUS

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I. INTRODUCTION

The past half a century has seen recorded a prodigious amount of practical and experimental research on induction of immunity to tuberculosis. A moderately effective vaccine was developed empirically some four decades ago; that today nearly the same vaccine is the best available attests to how little has been learned of how and by what portions of the tubercle bacillus tuberculoimmunity¹ is induced. The chronicity

¹ The term "tuberculoimmunity" will be used throughout this review to designate that refractoriness to infection which can be provoked in experimental animals and in man by injection of whole, living or killed tubercle bacilli and as is done traditionally with BCG. It is used in preference to "tuberculoresistance" or "resistance to tuberculosis" for the following reasons. Resistance connotes any of many ways a victim has of warding off an infection, several of these falling more properly under the scope of physiology than of immunology. Although to separate these fields of study often may be very difficult, one would tend to place variations in resistance to infection connected with nutritional factors or chemotherapy in the ken of physiology. On the other hand, tuberculoimmunity by its derivation includes itself specifically among immunologic phenomena. Unfortunately, the word "immunity" often provokes mental images of vaccinated animals completely refractory to infection with the microorganism against which they have been vaccinated, but a standing rule in immunology has been that protection derived from vaccination

of tuberculosis and technical difficulties that so often have been encountered in working with its causative agents are good reasons for slow progress in this research field, but several radically new techniques and marked improvements of old ones developed in the last dozen

against any disease can be defeated by severe enough infection (1a, 1b). When this happens, whether or not immunity is present can be measured only by comparing the severity of disease in vaccinated and unvaccinated animals. Many kinds of immunity (*e.g.*, against diphtheria) are difficult to overcome. Acquired immunity to tuberculosis, on the other hand, seems to be somewhat more easily overcome, but this is no reason to deny its existence. For example, the guinea pig, the favorite animal for tuberculosis research, can be fatally infected with as few as 10 virulent tubercle bacilli (1c). Yet, rarely do experimenters use less than 1000 times this dose to challenge their immunized animals, and usually this figure is closer to 100,000. It should not be surprising, then, that immunized guinea pigs which have been infected with 100,000 LD₁₀₀ numbers of tubercle bacilli should seem to be only "resistant" to this disease and not "immune." The chronicity of tuberculosis makes challenge infections approximating those that occur naturally in man rather impractical in the laboratory. But, statistical evidence in man himself has proved beyond reasonable doubt that vaccination with attenuated or killed tubercle bacilli does induce immunity in the broad classic sense as something which is not infallible but is specifically protective (1c, 15).

years are beginning to supply incisive data from which plausible hypotheses to explain tuberculoimmunity are germinating. The purpose of this paper is to hasten and aid such germination by presenting a summary and analysis of the most significant experiments related to tuberculoimmunity induction with bacillary constituents on the premises that since killed tubercle bacilli are immunogenic, one or more of their constituents can be shown to be chargeable with immunogenicity, and that if these ingredients can be isolated for study, important steps toward understanding tuberculoimmunity and related phenomena must quickly follow. Inasmuch as bacillary constituents are this review's primary concern, the reader will not find here detailed treatment of indirectly related subjects such as the immunogenicity of attenuated or killed tubercle bacilli. This essay is ordered according to the probable chemical categories under which the extracts are classifiable. Those which cannot be classified are discussed under "Combined Constituents." A section entitled "Heterologous Bacteria" has been found essential to a full exposition of the principle topic and constitutes this paper's final division.

II. POLYSACCHARIDES

Polysaccharides of varying chemical complexity and antigenic or haptenic activity have been isolated from tubercle bacilli (1e-3). Among the more purified polysaccharides, Seibert's high molecular weight polysaccharide II seems to be antigenic (1e), although it could owe its antigenicity to being complexed with a lipid (1e, 4). To date, the others have proved only haptenic (1e-3). The tuberculopolysaccharides administered by themselves generally are held not to be immunogenic, and considerable indirect and some direct evidence can be mustered to support this opinion.

The indirect evidence comes from extensive but unsuccessful attempts which have been made to immunize against tuberculosis with crude preparations such as old tuberculin and culture filtrates. These preparations are known to contain substantial amounts of polysaccharides (5-7). Their lack of immunogenicity is documented below under "Proteins."

As for direct evidence, Seibert (8) vaccinated rabbits repeatedly with each of her two purified culture filtrate polysaccharides (I and II) and then challenged them with virulent bovine

tubercle bacilli. The rabbits had not acquired immunity. Raffel (9) reports that guinea pigs intensively vaccinated with similar polysaccharides, usually extracted from culture filtrates but occasionally from defatted tubercle bacilli, developed no immunity.

In both of these series of experiments, positive control animals were vaccinated with living, attenuated tubercle bacilli (BCG). Among Raffel's animal groups were some injected with heat-killed tubercle bacilli. These, however, showed no increased resistance to the challenge infection. Since nonliving-vaccine controls were not used by Seibert, one cannot be certain that a small degree of tuberculoimmunity induced by the polysaccharides did not go undetected in her experiments as well as in Raffel's. However, later experiments in Raffel's laboratory ((10) and *unpublished data*) employing control groups developing resistance upon vaccination with nonviable bacilli or bacillary extracts support the previous finding that tuberculopolysaccharide is not immunogenic *per se* or when mixed with miscellaneous bacillary fractions.

In an excellently controlled experiment, Jespersen and Magnusson (11) observed two polysaccharides isolated from tubercle bacilli, and analogous to Seibert's fractions, to be incapable of immunizing field mice against bovine tubercle bacilli, irrespective of whether they were administered in buffer or in oil-in-water suspension.

Against these several experiments failing to show individual tuberculopolysaccharides capable of inducing immunity, as well as those described below in which the polysaccharide-containing tuberculins were used unsuccessfully, stands the so-far unrepeated work of Toda and Murata (12) exhibiting three apparently carefully done guinea pig experiments in each of which a moderate but definite degree of anti-tubercular resistance was provoked by injections of a tuberculin polysaccharide.

To prepare this polysaccharide, the filtered tuberculin was treated with acetic acid to lower its pH to 3.8 and remove proteins, and the resulting supernatant fluid was shaken with kaolin to remove polypeptides. The kaolin-treated fluid minus the kaolin then was treated with alcohol to precipitate the active polysaccharide.

In these same experiments, the protein and polypeptide fractions failed to induce resistance whereas the polysaccharide induced moderate,

and killed bacilli, good resistance against tuberculosis. If these data could be confirmed, they would be of utmost importance, for the active fraction does not seem particularly different from those which have been shown to have no activity.

The present reviewer knows of no succeeding published experiments by Toda with this polysaccharide. In view of the apparent lack of confirmation from other laboratories of this work, and the very strong evidence in opposition to it, tuberculopolysaccharides themselves cannot yet be given any credit for immunogenicity.

III. PROTEINS

Tuberculo-proteins command attention because of their role in tuberculin allergy (1e, 3, 13, 14), and because the relationship between tuberculin allergy and tuberculoimmunity still is enigmatic (9, 14-24, 140). Consequently, both crude and purified, they have been tested often for immunity inducing capacity. From the consistency with which experimenters have failed to detect immunogenicity while using adequate techniques, the conclusion that these proteins do not immunize seems justified.

One to twenty-seven injections of large amounts of proteins A, B, and C (Seibert (8)) from unheated tuberculin filtrate failed to increase resistance in rabbits to subsequent experimental tuberculosis. Guinea pigs could be made allergic by repeated intradermal injection of an ultrafiltration-concentrated and trichloroacetic acid-purified culture medium protein (TPT), but it gained them no protection (25). Intensive vaccination of guinea pigs with an unheated medium protein purified by repeated half-saturated ammonium sulfate precipitation provoked antibody formation but no resistance (9).

Neither by longer life after experimental tubercular challenge nor by macro- or microscopic evidence could immunity be observed in guinea pigs or rabbits treated with daily and increasing doses of medium tuberculo-protein, which by this treatment elicited skin hypersensitivity to itself (19). Since this was a skin sensitivity caused by circulating antibodies and since no immunity was detected despite this evidence of their presence, antibodies against tuberculo-protein must not be protective. Similar experiments performed by Krause (26) yielded agreeing results. From Japan, Toda and Murata (12) have

reported their failure to detect immunity in guinea pigs vaccinated with purified tuberculin protein or polypeptide fractions.

These experiments show that relatively purified medium protein is capable of calling forth antibody formation and immediate hypersensitivity but not immunity. For these fractions to contain small portions of polysaccharides has been common and contributes to the evidence acquitting the polysaccharides of direct responsibility for immunity induction. Thus, more evidence that neither protein nor polysaccharide is active *per se* comes in the form of experiments with crude protein fractions or tuberculin, the latter including both heated and unheated culture filtrates which have not been refined.

From a series of experiments reported some years ago, Corper (16) concluded, with good reason, that neither Seitz filtrates of medium growing virulent tubercle bacilli, highly concentrated filtrates, alum-treated filtrates, nor tuberculo-protein precipitated from such filtrates—all able to sensitize guinea pigs anaphylactically—could immunize these animals against virulent tubercle bacillary infection.

Two experiments from other laboratories serve as impressive examples of why this conclusion seems justified. In 1913, Klopstock (27) treated guinea pigs for 149 days preceding challenge infection with a total of 24 ml of old tuberculin. Repeating this kind of experiment with some improvements, Follis (28) injected guinea pigs on alternate days with 2 ml of culture concentrate for 6 weeks, infected the animals, and continued treatment for 44 more days. The total concentrate he administered was 130 ml. Neither worker found the guinea pigs particularly resistant to the challenge infection.

Field mice could not be immunized against bovine tubercle bacilli with purified tuberculin administered with or without oil adjuvant (11). A suggestion that bacillary culture filtrate administered in buffer was somewhat protective requires corroboration since statistically the suggestion carried little weight and since the same filtrate was not effective administered in the adjuvant employed.

Tuberculo-protein extracted directly from bacilli rather than from medium in which they have autolyzed conceivably might be less degraded and therefore immunogenic. Seibert and Fabrizio (29) found that such a tuberculo-protein extracted with urea from the H37Ra strain of

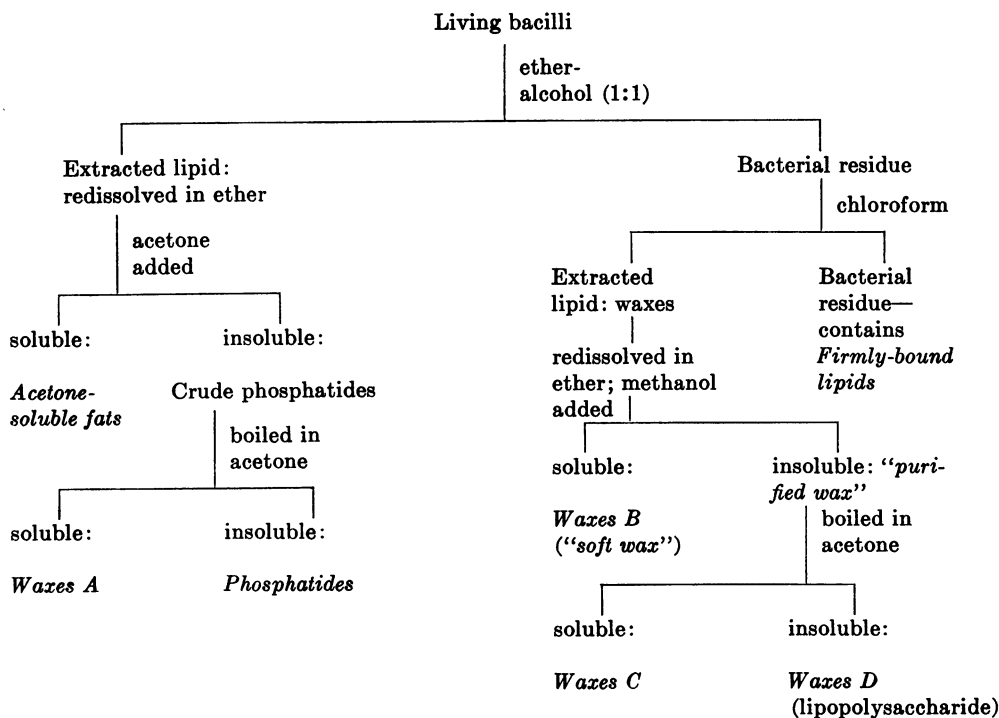
tubercle bacillus could, like medium protein, sensitize guinea pigs but, also like medium protein, evoked no immunity in them. According to Takeda and Kiuchi (30), Toda and other Japanese researchers have observed that proteins extracted from bacterial cells with dilute alkali afforded but slight resistance to tuberculosis. In unpublished experiments, Crowle, and Crowle and Raffel derived a crude proteinaceous fraction, soluble in borate-citrate buffer at pH 8.4, from mechanically disintegrated H37Rv and H37Ra strains of tubercle bacilli. This fraction, its purified proteins, and culture filtrate proteins of varying purity failed to elicit immunity in any of numerous carefully controlled experiments in which they were injected in water-in-oil emulsions into both guinea pigs and mice. The guinea pigs regularly formed antibodies against these fractions.

This ample evidence that tuberculo-proteins lack immunogenicity is not contradicted effectively by occasional independent reports in which proteinaceous fractions seemed to induce some resistance. Experimental repeated vaccina-

tion of rabbits with old tuberculin (19) seemed to induce a slight immunity to later infection, but comparison of this vaccinated group with a BCG-vaccinated control group shows this apparently increased resistance to be insignificant. A fraction extracted from defatted tubercle bacilli with 10 per cent barium hydroxide and used to vaccinate experimental animals intensively is said to have provoked a remarkable resistance to experimental tuberculosis (31). This extract was centrifuged to remove tubercle bacilli, a procedure which may have failed to remove fine particulate matter such as that found to be immunogenic by Youmans and co-workers (32).

Increased resistance in guinea pigs vaccinated with Tween 80-containing oil-in-water emulsion of *o*-aminophenol azotuberculin derivative (24) probably was due to protection by the emulsion itself since virtually the same preparation without antigen has been found to confer antitubercular resistance in field mice in another laboratory (11); the appropriate negative emulsion control was not included in these experiments.

TABLE 1
Fractionation of tubercle bacillary lipids (Asselineau (33))



Without serious exception, then, tuberculo-proteins derived from bacillary culture medium or directly from tubercle bacilli either by themselves or with the polysaccharides to which they usually are bound do not immunize against experimentally produced tuberculosis.

IV. LIPIDS

As much as 40 per cent of tubercle bacillary dry weight can be lipoidal (33), a fact which has not escaped curiosity in many laboratories. These lipids are responsible in part or entirely for many of the tubercle bacillus's peculiar physical and biological properties such as provocation of tuberculin allergy, immunologic adjuvant activity, induction of antibody formation, acid-fastness, hydrophobic characteristics, and possibly virulence (33-36). To find them involved in tuberculoimmunity, then, should be no surprise—and, indeed, this seems to be true.

Like both polysaccharides and proteins, the lipids have been experimented with in various states of purity. To avoid confusion, they will be discussed here according to their classification by Asselineau (33) and Asselineau and Lederer (35) which, in turn, is based on that of Anderson (37, 38) (table 1). This system categorizes the lipids empirically into acetone-soluble fats, phosphatides, waxes, and "firmly-bound" lipids. The first three classes of lipids can be removed from tubercle bacilli with neutral organic solvents, but the fourth is extractable only by harsher treatment, such as hot acid or alkaline alcohol, which fractures bonds between the lipids and polysaccharides or proteins (33). Lipoidal fractions isolated by procedures differing from those of the classic scheme can be placed fairly accurately in this scheme providing they are relatively pure and some of their properties are known.

A. Acetone-Soluble Fats

An ether-alcohol extraction of tubercle bacilli yields a crude fraction containing fats, phosphatides, and waxes. The phosphatides and waxes precipitate from an ether solution of the crude fraction when acetone is added, and the lipids remaining dissolved in the acetone-ether supernate constitute the "acetone-soluble fats." These fats also can be extracted directly from tubercle bacilli with acetone. They have been

credited with lowering native resistance to tuberculosis and with being toxic.

The acetone-soluble fats and waxes tested in tuberculous guinea pigs and rabbits by Boquet and Nègre (34) and Nègre and Boquet (39, 40) usually aggravated the disease. In these experiments, the lipids were administered after infection, that is, therapeutically.² Takeda and Kiuchi (30) administered acetone-soluble fat dissolved in paraffin oil to infected guinea pigs weekly without producing either beneficial or detrimental effects on the disease. Kubo *et al.* (41) obtained similar results also using the fats therapeutically.

The latter workers (41) tested the acetone-soluble fats for immunogenicity and found that they may lessen rather than increase anti-tubercular resistance. Toda and associates (42) could detect no immunity in experimental animals after their course of four injections totaling 5 to 7 mg of this fat. Yamasaki (43) found it to diminish resistance to tuberculosis in rabbits.

With one accord, then, experimental results declare tubercle bacillus acetone-soluble fats nonimmunizing. In fact, because they may decrease resistance, some workers prefer washing them out of bacilli with acetone prior to extracting possibly immunogenic lipid fractions (44, 45).

B. Phosphatides

Contrary to the acetone-soluble fats, the tuberculophosphatides frequently have been regarded as therapeutic and prophylactic anti-tubercular agents. These phosphatides can be extracted from whole bacilli with an ether-alcohol mixture. If this crude extract then is dried, redissolved in ether, and the solution treated with

² Since one class of lipids which has increased antitubercular resistance is effective therapeutically as well as prophylactically, the present writer must, to present a complete analysis of immunogenic potentialities of tuberculolipids, invoke the observation of Takeda and Kiuchi (30) that because of the chronicity of tuberculosis, the effects of therapeutically and prophylactically administered fractions very well may overlap and be due to similar mechanisms. This implies that both prophylactic and therapeutic data should be considered in studying the purported effects of some tuberculolipids in protecting against tuberculosis.

acetone, a mixture of phosphatides and waxes precipitates leaving in solution the acetone-soluble fats. Because acetone does not efficiently dissolve the phosphatides, subsequently boiling the precipitate in this solvent removes in solution a wax A fraction (33), leaving behind the washed phosphatides.

The classic fractionation from ether-alcohol extract provides purified phosphatide, but it is a phosphatide extractable from acetone-washed tubercle bacilli with methanol which has proved the most interesting immunologically. Originally, it attracted experimenters' attention because it could be used in serologic tests, but subsequently as *antigène méthylique* (34) it was shown to have *in vivo* antitubercular activity. As will be seen below, fractions closely related to this one also seem to elicit an as yet undefined kind of increased resistance to tuberculosis which differs importantly in several respects from classic immunity such as called forth by BCG vaccination (44, 47-50).

As the best known and most often tested of the resistance-inducing phosphatidic fractions, *antigène méthylique* deserves the most detailed scrutiny. Typically, it is extracted from autoclaved, acetone-washed, air-dried tubercle bacilli by exposing them for several days to pure methanol. It preserves activity well when kept in methanol, and although it may flocculate at low temperatures, warmth easily redissolves it. To be used as an antigen in serologic tests or for animal injections, its methanol solution is slowly added with vigorous mixing to an equal amount of water to form a very fine suspension which remains stable as the methanol is evaporated off at 48 to 50 C.

The methanol extract deserves its appellation *antigène*, for it can induce antibody formation against some of its constituents (34). Primarily a phosphatide, in several ways it resembles lecithin (34, 45), but it also contains some fats, waxes, and complex nitrogenous materials (34). These nonphosphatides may play roles in its antigenicity, for the phosphatide moiety itself diligently purified is merely haptenic, that is, it retains *in vitro* serologic activity but does not provoke antibody formation (51). The phosphatide constituent of *antigène méthylique* has not been proved but only suggested to represent the crude preparation's antitubercular capacity in animals (39). This thought must temper judgement of the following discussion.

By their numerous experiments with it, Boquet and Nègre (34) Nègre and Boquet (39, 40, 45, 56, 57), Nègre (47, 53-55), Nègre *et al.* (52), and Nègre and Bretey (58) have proved beyond much doubt that *antigène méthylique* used prophylactically can increase antitubercular resistance in rabbits and guinea pigs. They also indicate by these experiments, however, that such resistance is not necessarily equivalent to classic antitubercular immunity, although it could account for part of it. Classic tuberculoimmunity has certain characteristics which, whether they are directly connected with it or not, serve to define it. For example, its appearance is accompanied by emergence of tuberculin allergy (9, 13, 16-18, 22). Days to weeks must pass after vaccination before it appears (15, 49, 59-61), but once present it lasts for months, and usually years (15, 60, 62). As is documented in another section of this paper, to a certain degree it is specific. That these characteristics and others of classic tuberculoimmunity contrast with those of *antigène méthylique*-induced resistance will become evident.

Some experiments recently reported by Nègre (47) exemplify this antigen's protective activity. Guinea pigs and rabbits repeatedly injected with 5 ml aqueous suspension of the antigen, each milliliter corresponding to antigen extractable from 10 mg of dried bacilli, showed appreciably more resistance to tuberculous challenge 15 days after the last injection than did untreated controls. This acquired resistance, unaccompanied by tuberculin allergy, lasted for about four months—less than is expected of classic immunity (47, 59). This experiment typifies the apparently large quantities of methanol antigen required to immunize. One tenth to one hundredth as much killed tubercle bacilli easily suffices to induce classic immunity (63, 142). However, if phosphatide is assumed to be the only active ingredient of the preparation (55), this quantitative difference may be less striking, since the chemical equivalent of 0.5 mg lecithin was employed per injection (45).

Among the observations most suggesting that resistance evoked by methanol antigen basically differs from classic tuberculoimmunity are those that the methanol antigen is effective therapeutically. This extract can protect animals already infected (39, 40, 44), and it has been used for therapy in tuberculous human beings (56, 57). Since no evidence has been presented to

dissociate this therapeutic protection from protection induced by the extract before infection (*i.e.*, immunization), there is no reason to doubt that an induction period which is a characteristic of true immunization is unnecessary and, therefore, that the methanol extract is not immunizing, in the presently accepted sense of the term. Interestingly, regarding differences between classic immunity and that elicited by this extract, the extract has been observed to enhance BCG-induced immunity (47). Inasmuch as BCG is the most effective immunizing agent now available against tuberculosis, for the methanol extract to bolster such immunity suggests that it functions differently, a possibility proposed by Nègre who executed these experiments (47). For example, it may act by stimulating protective enzyme formation in the host (see below) or by detoxifying irritating substances in the tubercle bacillus (55). What part the resistance evoked by this antigen plays in classic tuberculoimmunity remains yet to be seen.

If tuberculophosphatide is the resistance-inducing moiety of *antigène méthylique*, then perhaps tuberculophosphatides prepared by other techniques should show similar protective activity. Among representatives of this bacillary constituent, the best known is the classic phosphatide of Anderson (64). However, experimentally supported opinions from various laboratories do not agree with regard to its immunizing powers. Kubo *et al.* (65) found that Anderson's A3 phosphatide fraction injected five times into rabbits at 2 to 3 day intervals for a total dosage of 5 mg was not only not protective against bovine tuberculosis but also possibly detrimental. Varying the inoculum from as much as 10 mg to as little as 10^{-8} mg did not express from it any immunogenicity. Therapeutically, also, it was ineffective. Much larger amounts of the same fraction (240 to 1040 mg) tested in a few rabbits in Sabin's laboratory (66) proved it, if anything, a weakening influence on antitubercular resistance. In guinea pigs, tested at this same laboratory, it had neither positive nor negative effects on resistance to tuberculosis. Raffel (9) could detect no increased resistance against heavy challenge infection in guinea pigs vaccinated repeatedly over 13 weeks with a total of 5 mg of this phosphatide or with a mixture of it and tuberculoprotein.

Contrary to the implications of this series of experiments that Anderson's tuberculophos-

phatide does not enhance antitubercular resistance, three laboratories in Japan report finding it protective. Toda and associates record that 5 to 7 mg of phosphatide administered to rabbits⁴ in four portions increased their resistance to tuberculosis without inducing tuberculin allergy (30, 42). Successful immunization of guinea pigs with this fraction alone or together with a polysaccharide was achieved again by Toda and Murata 5 years later (12). According to Takeda and Kiuchi (30), similar results have been reported by Kawamura. Comparison of the contrasting reports does not readily reveal reasons for their disagreement. Apparently, however, under certain presently undefined conditions Anderson's tuberculophosphatide may increase antitubercular resistance in experimental animals. Critical and exact repetition of successful Japanese experiments is required to resolve this disturbing discrepancy of results.

Data bearing on the resistance-inducing activity of *antigène méthylique* and, possibly, that of tuberculophosphatides recently have been published by Weiss and Dubos (49) who prepared and tested for immunogenicity in mice a crude extract apparently quite similar to that of Nègre (67). The Weiss-Dubos fraction is extracted with methanol from acetone-washed tubercle bacilli (44). Though not soluble in water, it suspends in it readily. Optimal immunogenic activity in mice is attained with this extract at several times the weight of killed tubercle bacilli needed to achieve the same immunizing effect: 4 mg of the extract approximately equal the protective power of 1 mg of the bacilli. Just as Nègre found his methanol antigen to enhance BCG immunogenicity (47), Weiss and Dubos observed their extract to promote the immunogenicity of a comparable human avirulent tubercle bacillus, H37Ra (49). The postulation prompted by such observations that these extracts act synergistically with a whole bacillary vaccine or as adjuvants (47) seems logical; on the other hand, their rather nonspecific protective activity (68, 69) may account for these results which would be quite similar if a slowly released antitubercular drug (*cf.* 46) were used in their stead. Thus, the short lived resistance induced by the methanol antigens can be lengthened and strengthened by their injection in water-in-oil emulsions, perhaps because of the long term depository action of these emulsions (71), as well as their adjuvant

properties, from which small amounts of the antigen gradually are released over a long time.

Two other laboratories have expressed experimental interest in the immunogenicity of methanol extracts of tubercle bacilli. Data from one of these (72) are incomplete and consequently provide no information which can be contemplated profitably here. In the other laboratory, Youmans ((73) and *personal communication*) was not able to increase antitubercular resistance in mice in any of several separate experiments employing batches of methanol extract prepared according to the Weiss-Dubos procedure. Since details of these experiments are not yet published, possible reasons for the disagreement between these two laboratories cannot be discussed here except to say that they may lie in the considerably different techniques commonly used in the two laboratories.

A digestion of these data on the possible immunogenicity of tubercle bacillus methanol extracts and their supposed active constituents, the tuberculophosphatides, indicates that these preparations probably do enhance antitubercular resistance, but that this is not likely equivalent to tuberculoimmunity. However, since this kind of resistance may be important practically and, although not equivalent to tuberculoimmunity, might take some part in it and certainly in its study, speculation on how it functions should prove interesting.

Some tubercle bacillus lipids have been shown to be toxic or irritating to both normal and infected animals (4, 33, 34, 50, 54, 57, 66, 75, 77, 78, 82). Consequently, protection against the most offensive of these might help cope with tuberculosis (74). Take, for example, the following observations. Native resistance of various animal species and their organs to tuberculosis appears to be correlated with phosphatidase activity (74-76). Tuberculophosphatide provokes tubercloid tissue formation (77). It may kill cells which ingest it (78), and it can interfere with an enzyme responsible for repairing injured tissue (50). Therefore, that phosphatidase activity should reflect native antitubercular resistance is not illogical. If an injected substance were to increase or initiate this activity in animals, it could be called immunogenic.

The work of Gerstl and Tennant (48, 74, 75, 79) supplies this logical deduction with some

experimental support. According to these experiments, intraperitoneal injections of tuberculophosphatide which caused characteristic, pathological changes within the peritoneal cavities of normal rabbits were relatively harmless to rabbits previously vaccinated either with tuberculophosphatide or with the sodium salt of cinnamoyl glycerol phosphatidic acid, a synthetic lipid resembling tuberculophosphatide. Intravenous vaccination with the synthetic salt actually prevented the usual tubercloid tissue formation in the peritoneal cavity. These workers hypothesized that animals with preliminary exposure to tuberculophosphatide later can destroy it more quickly than unvaccinated animals by virtue of stimulated enzymatic activity and thereby are protected against its unfavorable effects.

Direct evidence of any relationship between these observations and the apparent protectiveness of tuberculophosphatides, crude or refined, is a golden apple yet to be plucked. However, circumstantial evidence continues to be suggestive. For instance, the organs and serum of either BCG-immunized guinea pigs or natively resistant rats contain several times the tuberculolipase and tuberculophospholipase activity of organs and serum from unimmunized guinea pigs (80).

Unfortunately for a complete understanding of how methanol extracts of tubercle bacilli elevate resistance, their activity seems more than simple or single as is so clearly established by recent experiments from Dubos' laboratory (68, 69). These show that such an extract can protect mice against nontubercular infection or decrease resistance to it, depending upon the time relative to infection that it is administered.

Before beginning a discussion of other tubercle bacillary lipids as immunogenic agents, the writer would like to remark that since the tuberculophosphatides are soluble in commonly used organic solvents, excepting acetone (33), they can be expected to occur in appreciable quantities in most unpurified organic solvent tubercle bacillary extracts. Hence, their probable antitubercular activity must be accounted for prior to crediting any such extract with immunogenicity.

C. Waxes

The four major waxy fractions of tubercle bacilli tend to overlap in composition (81), but

have been separated for practical purposes by their differing solubilities in various organic solvents (33, 35). They are designated by the letters A, B, C, and D.

The A waxes are extracted from tubercle bacilli with an ether-alcohol mixture and can be dissolved from the dried residue of this extract with ether alone. Acetone added to this ether solution precipitates both them and the phosphatides which were extracted at the same time. Subsequently boiling the crude precipitate in acetone dissolves the waxes but not the phosphatides. The wax A fraction contains notable quantities of various esters of phthiocerol with fatty acids as well as the free fatty acid, mycolic acid.

The waxes B, C, and D are leached with chloroform from bacilli already extracted with ether-alcohol. When the chloroform extract is dried and redissolved in ether, and methanol is added to it, a portion precipitates. This is Anderson's "purified wax" (waxes C and D). The supernatant fluid when dried yields Anderson's "soft wax," a lipid semisolid at room temperature and which, by more recent nomenclature, is wax B. The "soft wax" is a complex mixture of waxes and glycerides, also containing a small amount of free mycolic acid (38, 81).

Waxes C and D are separated by extraction with boiling acetone since this treatment dissolves wax C but not wax D. Wax C has several constituents in common with both A and B waxes but not necessarily in the same relative quantities. Wax D differs from the other three waxes in consisting almost entirely of a polysaccharide ester of mycolic acid containing both nitrogen and phosphorus in addition to the expected elements.

By their own techniques, Bloch and Choucron have isolated two tubercle bacillus lipid fractions of considerable interest. These are, respectively, "cord factor" and "Pmko." Fortunately, both are well enough characterized chemically to identify them by the classic fractionation scheme. "Cord factor," extracted from young bacillary cultures with petroleum ether (82), occurs primarily in the wax C fraction (83). "Pmko," extracted from tubercle bacilli with warm paraffin oil (88), probably is identical to wax D (84, 143).

The bacillary lipids have been subjects of many well described chemical and biological experi-

ments. Amazingly, despite their several obvious biologic activities, their possible significance in tuberculoimmunity has received, by proportion, almost no attention, according to available published information. The unfortunate tendency of experimenters to report a minimum of their valid but negative results may account for this apparent lack of interest. Consequently, one must not be surprised at the often meager data on hand for discussion in the following paragraphs.

The possible role of waxes A and B in immunity-induction is virtually unstudied. Hints that they are not immunogenic come from Raffel's (85) and *personal communication*) tuberculin allergy experiments in guinea pigs, some of which were extended into tests for acquired immunity. However, because these experiments were intended to study tuberculin allergy, their information on induction of immunity is not complete enough to dissociate waxes A and B from this activity. Since wax A is a significant constituent of ether-alcohol extracts of tubercle bacilli, the findings of Crowle and Raffel (*unpublished data*), in experiments designed to study immunization rather than tuberculin allergy, that this crude extract failed by itself to immunize mice suggest more strongly that wax A is not active in this complex mixture of bacillary lipids.

More definite conclusions are justified regarding waxes C and D. As the combination represented by Anderson's "purified wax," they have shown no significant immunogenicity. Hoyt and associates (86) found that this mixed lipid's apparent ability in their hands to induce immunity in mice was due to heavy bacillary contamination, for when the bacilli were removed by centrifugation, "purified wax" lost all such activity. In Raffel's laboratory, this wax administered either alone or together with "defatted" tubercle bacilli did not immunize guinea pigs to heavy challenge infection despite inducing tuberculin allergy (9). The lipopolysaccharide moiety of "purified wax" (wax D) was shown several times not to induce immunity in guinea pigs (Raffel, *personal communication*). Results from similar experiments done in two Japanese laboratories (31, 42) agree that "purified wax" confers no antitubercular protection.

A conclusion that a single intradermal injection of 1 mg of this wax in paraffin oil may have produced "some protection" in guinea pigs in

which it provoked a low degree of tuberculin allergy (87) does not seem warranted from recorded numerical and graphic data. Median survival for these pigs was about 6 weeks; for the unvaccinated controls, it was between 5 and 6, whereas among comparable bacilli-vaccinated animals it was between 9 and 10 weeks.

Choucroun's reports (88-90) that Pmko or the liquor yielding it are immunogenic in part disagree with these results, for Pmko is wax D purified from a paraffin oil extract rather than a chloroform extract of tubercle bacilli. Paraffin oil was used for initial extraction presumably because of its well known adjuvant activity (91) which, since it often has meant the difference between success and failure in immunizing animals with killed tubercle bacilli (10), suggested that it might enhance immunogenicity by extracting and making more readily available to the proper host tissues the immunogenic constituent of tubercle bacilli (91). Choucroun's experiments (88, 92-94) prove clearly that the crude paraffin oil extract and its Pmko derivative are most interesting immunologically, but they are not convincing proof of the fractions' immunogenicity.

In 1947, Choucroun (88) reported immunizing guinea pigs with Pmko. Data from this paper intimate that Pmko used then contained biologically important nonlipid bacillary constituents, notably tuberculoprotein. An article she published in 1949 supports this intimation (89). According to the 1949 paper, her "sensitizing fraction," one able to induce tuberculin allergy, contains both Pmko and tuberculoprotein. With considerable trouble, it could be freed of Pmko but thereupon lost tuberculoallergenic activity. Conversely, purified Pmko could not sensitize to tuberculin unless mixed with a small amount of tuberculoprotein. Since the Pmko used in her first experiments (88) induced tuberculin allergy, it most likely was not equivalent to purified wax D but was a lipid-protein-polysaccharide complex or mixture. Inasmuch as Choucroun has not reported details of any immunity experiments employing highly purified Pmko, the immunogenicity of this fraction as a counterpart of wax D is not established, and there is no contradiction of experiments which have failed to attribute this wax with immunogenic activity. This, of course, does not mean that either unrefined Pmko or crude paraffin oil

extract may not be able to induce immunity. This point will be discussed below.

The immunogenicity of wax C individually has not been tested. Philpot and Wells (95) tested Bloch's "cord factor," a component of wax C with some remarkable biologic properties, in a small guinea pig experiment but did not find it immunogenic.

Thus, from *in vivo* experiments available for study, it seems that waxes C and D do not account for the tuberculoimmunity induced by whole bacilli, and wax A probably is inactive. The status of wax B is unknown. *In vitro* (tissue culture) experiments still are too little tried and understood for discussion here; data which they have contributed have been inconsistent (10).

V. COMBINED CONSTITUENTS

Of the purified bacillary constituents which have been tested for immunogenicity, only one, the phosphatide, has proved likely to be active alone, and this one seems to induce a resistance different from tuberculoimmunity. Single fractions might by themselves be unable to immunize but become competent in combination with others just as a combination of tuberculoprotein and tuberculolipopolysaccharide is ideal for inducing delayed ("infection") allergy (96, 97). A good deal of our information on this aspect of immunity induction comes from tests with crude preparations known to contain two or more of the simpler fractions.

Raffel has studied induction of tuberculin allergy in guinea pigs by simultaneous administration of various bacillary constituents. In many of these experiments, the vaccinated guinea pigs were tested for resistance to tuberculosis at the end of allergy experimentation. Thus, he has accumulated considerable suggestive data on the probable nonimmunogenicity of various antigen combinations. One such series of experiments has been reported in detail (9) and indicates that guinea pigs intensively vaccinated with "defatted" tubercle bacilli and either "purified wax" or tuberculophosphatide, or with a high molecular weight culture medium tuberculoprotein together with the phosphatide were not immunized. In a more recent paper (10), Raffel summarizes work in his laboratory, stating that combinations of protein with crude wax and various wax fractions, the phosphatides, and a tuberculopolysaccharide have failed to immunize

guinea pigs. These data confirm the conclusions reached above that individually and in certain combinations "purified wax," tuberculo-protein, and tuberculopolysaccharide are nonimmunogenic. They do not support the contention that tuberculophosphatides may be protective. However, Raffel followed conventional rules of vaccination which, as has been suggested by the discussion of methanol-extracts alone, might not succeed in inducing the protection peculiar to the crude phosphatides. The point of primary interest in Raffel's experiments is his ability to induce tuberculin allergy and antibodies in guinea pigs by using protein-wax D (lipopolysaccharide) vaccines without eliciting tuberculoimmunity. These findings have been repeated often and under varying experimental conditions (Raffel, *personal communication*) and provide strong evidence that either or both of these purified constituents do not induce significant tuberculoimmunity. The significance of no immunization by the "defatted" bacilli is not certain, since complete but killed bacilli used in the same experiment (9) also failed to immunize.

Takeda and Kiuchi (30) injected guinea pigs twice weekly with a mixture of phosphatides, "purified wax," and acetone-soluble fats—all produced by Anderson's method—and tuberculo-protein, and infected them a month after the first injection when they had developed tuberculin allergy. Somewhat inconsistently, these animals showed a slight degree of increased resistance to tuberculosis, but since similarly infected guinea pigs treated during infection with this mixture showed greater resistance, the meaning of these results is unclear. Perhaps the protection elicited was of the kind induced by *antigène méthylique*, or perhaps, as Takeda and Kiuchi hypothesize, therapeutic and prophylactic effects of some of these bacillary constituents overlapped, acting by the same mechanism. These experiments confirm Raffel's conclusion that the bacillary constituents which induce tuberculin allergy do not effect immunity.

Experiments testing crude fractions or degraded tubercle bacilli for immunogenicity might, by deduction, disclose whether combined bacillary elements are active when single elements are not and, if so, what these or their properties might be.

Tubercle bacilli have immunized after being heated for various periods at different temper-

atures, including autoclaving (60, 63, 98-106). Although excessive ultraviolet irradiation probably destroys immunogenicity (107), the bacilli can be killed by ultraviolet and yet retain their activity (60, 63, 100, 107-110); cathode ray-killed bacilli have been used to immunize guinea pigs very efficiently (63). The bacilli have been treated with such chemicals as urea (100, 111-113), formol (100), phenol (49, 99, 114, 139), nitrous acid (115), hydrochloric acid (116), sodium hydroxide (116), or ethylene oxide (61) and retained varying degrees of immunogenicity. Likewise, they have been extracted with sundry organic solvents (60, 102), yet remained active though dead (117). By deduction, these data indicate that tubercle bacilli need not be living to provoke resistance and their immunogenic ingredient(s) is either very hardy or well protected in the bacillary structure.

Destructive measures against specific bacillary fractions might be as informative in trying to identify the active constituents as experiments using extracts. For example, Crowle (*unpublished experiments*) found a commercial sodium hypochlorite bleaching solution to diminish greatly the immunogenicity of entire tubercle bacilli—far more than could be attributed to its bactericidal effect. *In vitro* experiments showing that this solution rapidly destroyed tuberculo-protein antigenicity together with information from *in vivo* experiments were interpreted to implicate tuberculo-protein as an essential part of the immunizing complex. Other experiments utilizing entirely different approaches so far have corroborated this interpretation (Crowle and Raffel, *unpublished data*).

Single solvents can extract very heterogeneous substances from tubercle bacilli. A paraffin oil extract, for example, may contain substantial amounts of lipids, proteins, and polysaccharides; and this extract proves to be one well worth discussing, for Choucroun finds it immunogenic (88). Choucroun's experiments with this substance by themselves are not yet complete enough definitely to establish its effectiveness. However, Yanagisawa and collaborators (118) prepared a paraffin oil extract using Choucroun's method and observed that it both immunized guinea pigs and induced tuberculin allergy in them. The extract essentially was equal to BCG vaccine in these respects. Kajihara (141) obtained immunity and tuberculin allergy in guinea pigs injected in-

tracutaneously with 0.1 ml of this type of extract. These experiments strongly imply, then, that in this very crude extract might be found the substances which induce tuberculoimmunity.

Unfortunately, however, paraffin oil extracts prepared in different laboratories seem, for unknown reasons, to differ in immunogenic ability. Takeda and Kiuchi (30), for example, state that experimenters in Japan generally have failed to obtain results agreeing with Choucroun's findings. In the United States, Crowle (63) could detect no immunogenicity or tuberculin allergenicity in guinea pigs for a paraffin oil extract prepared according to Choucroun's method, except that the extract was decontaminated by high-speed centrifugation instead of filtration.

One observation seems consistent in all these reports, and that is that when the extract elicits tuberculin allergy it also provokes immunity. Since, as has been shown above, the extract contains the lipid equivalent of wax D and this wax and tuberculoprotein can induce tuberculin allergy when injected together (14), the extract must also contain tuberculoprotein. Therefore, failure of Crowle's preparation to immunize may in part have been owing to lack of protein in his preparation. Paraffin oil is an ill-defined mixture of hydrocarbons and may differ in composition according to its source. The hypothesis that Choucroun's own experimental results have not always been consistent with supposedly identical preparations has been attributed to just such solvent variations (91). Perhaps, then, disagreement in immunization results are due to the ability of some paraffin oils to extract tuberculoprotein (or any other essential element) along with the various lipids which others are unable to do. Further experiments are urgently needed to test this hypothesis, for if a paraffin oil or chemically defined hydrocarbon can be found which invariably will extract immunizing substances from tubercle bacilli, a great step will have been taken towards defining the immunogenic constituents of these bacilli.

A crude extract prepared by Smith *et al.* (119) using chloroform as primary solvent has shown immunogenic capabilities. This extract was passed repeatedly through filter paper, dried, redissolved in a 1:1 mixture of methanol and chloroform, from which a precipitate could be formed by cooling the solution to -30°C . The

mother liquid and precipitate immunized guinea pigs, and the former also protected rats. The supernatant fluid, on the contrary, actually may have increased susceptibility to tuberculosis. However, a later opinion from the same group of experimenters is that immunogenicity may have been due to bacillary contamination (102). Filter paper is known not to be reliable in removing mycobacteria quantitatively from organic solvents (117). The same possibility of bacillary contamination influencing results unhappily also may apply to subsequent experiments of Smith and Kubica (102), which occasionally demonstrated immunity in guinea pigs vaccinated with fractions that had been extracted from ethanol-washed tubercle bacilli with methanol-chloroform, or, on occasion, with subfractions precipitated from solution at low temperatures. Bacillary contamination was suggested as a misleading factor by these workers when they observed that percolating an active fraction through a chromatographic column removed its immunogenicity, presumably by removing bacilli.

From an ether-alcohol tubercle bacillary extract which had been Berkefeld-filtered in ether solution, Kropp and Floyd (120) precipitated a crude lipoidal-carbohydrate with 95 per cent ethanol. According to common qualitative tests, this extract contained no protein or protein-nitrogen. Given subcutaneously to guinea pigs in a series of injections preceding challenge, the extract seemed to immunize against tuberculosis. However, a subsequent guinea pig experiment testing it both therapeutically and prophylactically was less successful. As the authors suggest, these experiments are too limited to call this fraction immunogenic.

Widström's experiments using vaccines prepared from ground bacilli and consisting of their insoluble portion, their soluble portion, or both together represent the beginning of a systematic approach to isolating the bacillary immunogenic constituents by progressively refining active fractions. Unfortunately, Widström apparently has not continued in this vein, and the experiments on record (116) are too limited and variable in their results to supply information of much value in discovering the immunizing antigens.

Crowle (63) tested preparations of physically disrupted tubercle bacilli similar to Widström's and found cellular constituents, soluble in the

borate-citrate buffer employed, to induce no immunity in guinea pigs while inducing copious antibody formation and some tuberculin allergy. The insoluble residue or bacillary hulls, on the other hand, elicited statistically significant immunity, moderate antibody formation, and consistent tuberculin allergy. Subsequent experiments (Crowle and Raffel, *unpublished data*) showed that simultaneously injecting a lipid extractable from the active residue together with either the crude soluble fraction or protein derived from it resulted in tuberculoimmunity. Neither the lipid nor the proteinaceous fraction were active alone. These and related experiments have indicated that at least two chemically distinct bacillary constituents must act together to immunize against tuberculosis.

By the same general approach of disrupting tubercle bacilli, Youmans and co-workers (32, 121) have isolated from H37Ra and BCG strains of tubercle bacilli definable but chemically heterogeneous particles which immunize mice. These presumed mitochondria have enzymatic activity and originally were thought to owe their immunogenicity primarily to this (32), but the later experiments do not entirely support such an idea (121). The particles' chemical composition (considerable phospholipid, some protein, minor amounts of some other chemicals, no deoxyribonucleic acid) and their ability to retain a slight immunogenicity after autoclaving make credible attributing their activity to the combined immunizing effects of their constituents. Essentially, then, these experiments show that a chemically complex particulate fraction possibly corresponding to bacterial mitochondria, which can be extracted physically from entire, living tubercle bacilli, is able to immunize mice against tuberculosis as effectively as the whole bacilli themselves. An interesting question posed by these observations is whether bacillary mitochondria represent all the immunizing potency of tubercle bacilli or whether other portions also are active. Answering this question might yield information vital to understanding how anti-tubercular immunity functions against the invading bacteria.

In summary, immunity experiments employing crude bacillary extracts seem to imply that failure of the single purer preparations to induce tuberculoimmunity may be because more than one antigen must be injected for effect. Failures

probably seldom are due to chemical or physical treatments employed in preparing various extracts, for the active constituents seem to be rather sturdy. The paraffin oil extracts have immunized only when also sensitizing to tuberculin, and these active extracts are known to contain lipids, polysaccharides, and protein; one preparation which lacked protein, however, failed to immunize. Crude extracts of disrupted tubercle bacilli can be immunogenic and there are indications that their activity is due, in biological terms, at least partly to mitochondria-like particles, or, in chemical terms, to the combined activity of a tuberculoprotein and a tuberculo-lipid.

VI. HETEROLOGOUS BACTERIA

Offhand, consideration of the capabilities of bacteria other than tubercle bacilli to immunize against tuberculosis would seem misplaced in the present essay. However, this is necessary because the specificity of classic tuberculoimmunity is less than that of several well known humoral antibody immunities, and because the antitubercular resistance induced by some tubercle bacillary constituents may be nonspecific and therefore not fit even a broad definition of acquired immunity. In trying to identify the immunizing constituents of tubercle bacilli, it is necessary to recognize what kinds of resistance are being elicited by test antigens and how these compare with common concepts of classic tuberculoimmunity and classic humoral antibody immunity.

The high specificity of humoral antibody immunity is exemplified by failure among types of the same species of *Diplococcus* to immunize against each other. For practical purposes, only a single kind of antigen immunizes against pneumonia and the acquired immunity is solely due to antibodies formed against this antigen (14). This high specificity does not obtain among mycobacteria since different varieties (*e.g.*, bovine, human, murine) or species (*e.g.*, *phlei*, *balnei*, *ulcerans*, *tuberculosis*) cross-immunize with varying degrees of success (60, 100, 110, 122-127). In view of the probability that more than one kind of antitubercular resistance is induced by more than one bacillary constituent, this is not so strange as it might at first seem. Something of this kind may account for acquired immunity to cholera, for an example of the better understood acquired immunities (14). However, how does the

fact that taxonomically completely unrelated bacteria can induce effective antitubercular resistance coincide with our knowledge of acquired immunity?

On several occasions, experimenters have shown that vaccination with organisms other than mycobacteria enhances animal resistance to tuberculosis. Of several bacterial species they tried in guinea pigs, Nukada and Ryu (128) found a mixture of typhoid bacilli and gonococci best in this respect. Several years later, Nukada and Utsunomiya (129) extended these experiments to mice in which autolysates of these gram-negative bacteria proved even more effective. Waaler (130) observed that guinea pigs vaccinated intraperitoneally with typhoid-paratyphoid vaccine (TAB) became locally protected, showing more rapid disposal of attenuated bovine tubercle bacilli (BCG) injected into the same body cavity than occurred in previously untreated guinea pigs. Dubos and co-workers (131) found pertussis vaccine nonspecifically protective against tuberculosis in mice. The most successful experiments of this kind recently were reported by Nyka (132), and their results and analysis together with those from other experiments offer some ideas of why this kind of apparently nonspecific antitubercular resistance can be induced and how it is related to tuberculoimmunity.

According to these experiments (132), mice infected with either virulent or avirulent *Brucella abortus* and later infected with virulent tubercle bacilli showed resistance to tuberculosis quantitatively equal to that developing after vaccination with tubercle bacilli. However, this resistance seems to differ from tuberculoimmunity in at least two important respects. Immunization with *Brucella* was highly effective only if vaccination was intravenous; intraperitoneal or subcutaneous vaccination offered progressively less protection against tuberculosis. Attenuated tubercle bacilli, by contrast, immunize equally effectively by these three routes (60, 86, 126, 133-135). A second obvious distinction of *Brucella*-induced immunity to tuberculosis is that it did not decrease with passage of time as it has been observed to do in the same species of mice vaccinated with attenuated tubercle bacilli (60).

These observations lead to the reasonable explanation, proposed by Nyka (132) and commented upon favorably by Nukada (136) on the

basis of his own experience to explain such results, *i.e.*, that antitubercular resistance induced by *Brucella* depends upon the common route of infection shared by this and the tubercle bacteria. During primary infection, the host tissues contacting *Brucella* somehow are conditioned to resist subsequent infection of any kind by that route. Thus, the phagocytes resist destruction during secondary tubercle bacillary invasion and may actually become more able to destroy the tubercle bacilli.³ This is why the route of primary infection is so important to heterologous vaccination as pointed out particularly by Waaler's experiments with typhoid bacilli and mentioned previously (130). In this connection, it is interesting that the heterologous bacteria which increase resistance to tuberculosis are, like tubercle bacilli, found intracellularly during some phase of host infection (132, 138). *Brucella*-elicited resistance decreases little if any as time passes because a chronic, long-lasting infection establishes itself in the mice, apparently continually stimulating their native protective mechanisms (132).

VII. SIGNIFICANCE FOR MECHANISMS OF TUBERCULOIMMUNITY

Such data as these verify that the study of tuberculoimmunity is complicated by the extreme difficulty of defining exactly what is meant by the term and of distinguishing it in experiments from types of antitubercular resistance induced nonspecifically by vaccination, which may be due to such stimulated protective properties of the body as properdin (131). Although at some time in the future tuberculoimmunity may be proved to be a composite of these phenomena and not justifiably thought of as a specific resistance acquired in response to specific microorganisms, for practical purposes of comparison, discussion, and experimentation it seems best to risk contriving a workable definition of tuberculoimmunity from the data which have been gathered and analyzed here.

The model of tuberculoimmunity is induced by vaccination with attenuated tubercle bacilli such as BCG. The injection of a small amount of either

³The *in vitro* experiments of Elberg and colleagues (137) demonstrating cross-resistance between *Brucella melitensis* and tubercle bacilli in rabbit monocytes tend to support this concept with direct experimental evidence.

living or dead tubercle bacilli of this kind by any of various parenteral routes can evoke, after a definite induction period, a long-lasting though gradually weakening resistance against tuberculosis and, to a lesser extent, diseases caused by mycobacteria closely related to tubercle bacilli. It usually, but not necessarily, is accompanied by tuberculin allergy. By contrast, other types of vaccination-acquired resistance to tuberculosis may require large amounts of inducing antigen, may take effect even if the antigen is administered after tubercular infection, usually require special vaccination schedules and routes for optimum effect, are likely to last only a short while, and are not related to tuberculin allergy. Differentiating one kind of antitubercular resistance from the other seems to be necessary to avoid misunderstanding in any studies of the immunizing constituents of tubercle bacilli.

Although tuberculoimmunity in some ways resembles better understood kinds of immunity (those due to circulating antibodies), it also differs significantly. Like humoral antibody immunities, it requires an induction period, its potency can be augmented within certain limits by variations in the vaccination procedure, it has specificity, and effectiveness diminished by time can be restored or boosted by supplementary vaccination. It differs most glaringly in apparently not being associated with humoral antibodies; tuberculoimmunity never has been transferred successfully from vaccinated to unvaccinated animals of the same or different species using techniques known to be successful in transferring protective humoral antibodies. Preliminary studies (144, 145) suggest that it might be transferable using whole cells from immunized animals rather than serum. Although tuberculoimmunity has specificity, this seems to be considerably less than that of humoral antibody immunities.

Whereas the mechanism of humoral antibody immunity is a straight-forward one, manifestations of which can be seen in the test tube when antibody combines with and neutralizes some antigenic constituent vital to the pathogenicity of the invading microorganism, the mechanism of tuberculoimmunity at present is far from clear. Apparently, cells of immunized animals which ingest tubercle bacilli either can prevent bacillary multiplication or may actually destroy the bacilli. How these cells do this might be under-

stood if their immunization could be explained. Proposed hypotheses can be divided broadly into two categories: either the cells actively affect ingested bacilli or they hinder the bacilli passively. Space limitations allow mention of only two broad hypotheses as examples. Host cells might become able actively to destroy tubercle bacilli by exposure to a bacillary component(s) which induces adaptive enzyme formation in them, the new enzyme being capable of destroying this component. On the other hand, the resistant cells might become able to check bacillary multiplication (and spread of disease) passively by having had their metabolic rate or end products changed by immunization so that they would deplete their immediate area of an element metabolically essential to tubercle bacilli (e.g., oxygen), or would produce or accumulate as a by-product of their altered metabolism some substance toxic to the bacilli (e.g., lactic acid).

Speculation on the mechanism of tuberculoimmunity will rest on firmer ground when true tuberculoimmunity can be induced with a definable bacillary extract. Formidable barriers lying in the way of this accomplishment seem about to tumble. Their fall will mark the beginning of a true and most profitable understanding of a type of immunity which has puzzled three generations of researchers.

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