THE TUBERCULOIMMUNOGENICITY FOR MICE AND GUINEA PIGS OF MYCOBACTERIUM BOVIS INACTIVATED WITH BETA-PROPIOLACTONE COMPARED TO THAT OF BCG, PHENOL INACTIVATED CELLS, AND YOUMANS' EXTRACT

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ABSTRACT

Onyekwere, Okpo, O. (Michigan State University, East Lansing, Michigan). The tuberculoimmunogenicity for mice and guinea pigs of Mycobacterium bovis inactivated with beta-propiolactone as compared to that of BCG, phenol inactivated cells, and Youmans' extract. 1962.- Studies were made of the inactivation of Mycobacterium bovis with beta-propiolactone (BPL) for the preparation of a bacterin. A final concentration of 0.1% BPL was required to inactivate 3×10^7 cells per ml distilled water if incubated 2 hr at 37 C and the pH maintained at 7.6 ± 0.4. A safe effective bacterin was prepared with 0.4% BPL under the same conditions. The greater concentration provided a satisfactory safety factor. Washing the inactivated cells 4 times with distilled water removed toxic products due to the BPL treatment. The tuberculoimmunogenicity was measured by the difference in the percentage of the vaccinated and unvaccinated experimental animals surviving after challenge. When two-thirds of unvaccinated mice had died, 72%, 69% and 58% of BCG, BPL-bacterin and phenol-bacterin vaccinated mice survived, respectively. When two-thirds of unvaccinated guinea pigs had died, 100%, 90% and 80% of BCG, BPL-bacterin and phenol-bacterin vaccinated guinea pigs survived, respectively. Washing the phenol and BPL bacterins with acetone did not improve their efficacy. The BPL-bacterin induced tuberculin sensitivity in guinea pigs. An extract was prepared from M. bovis which conferred tuberculoimmunity to guinea pigs assumed to be

similar to that conferred to mice by Youmans' extract of *M. tuberculosis*.

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INTRODUCTION

Tuberculoimmunity is that refractoriness to infection with the tubercle bacilli which can be induced in man and animals with viable or killed organisms (Crowle, 1958a). It is regarded as an immunologic phenomenon but it is differentiated from the classical immunity associated with circulating antibodies. Specific antibodies are elicited in various hosts by the tubercle bacilli but no immunity is conferred by the antibodies.

Since the discovery of the causal agent of tuberculosis, the immunogenicity of living and killed tubercle bacilli has been investigated. Pertinent literature is abundant and contradictory. It is generally accepted that an attenuated living strain of Mycobacterium bovis, the Bacillus of Calmette and Guèrin, confers the greatest specific protection. Owing to the inherent dangers in the use of any bacterial vaccine, an equally effective bacterin is more desirable.

Beta-propiolactone has been used for the sterilization of serum and plasma fractions and the inactivation of viruses and bacteria other than the mycobacteria. It is non-toxic for man and animals in low concentrations which inactivate microorganisms. It reportedly does not denature proteins and therefore, the antigenicity should be retained in a non-toxic bacterin. To determine if the tuberculoimmunogenicity of M. bovis is retained after the inactivation

by beta-propiolactone, a comparison has been made of the specific protection afforded mice and guinea pigs by the administration of the following: (a) M. bovis inactivated with beta-propiolactone, (b) M. bovis inactivated with phenol, (c) BCG vaccine, and (d) an extract of M. bovis prepared by the method of Youmans (1960).

HISTORICAL REVIEW

Soon after the discovery of the tubercle bacillus (Koch, 1882), the concentrated culture filtrate, Old Tuberculin, was used prophylactically and therapeutically (Koch, 1890). The product proved to be ineffective and, in fact, so hazardous that it momentarily jeopardized Koch's international reputation. Subsequently, it was found to be of diagnostic value.

Tuberculoimmunity afforded by BCG. The first vaccine investigated for immunization against tuberculosis was the Bacillus of Calmette and Guèrin (BCG). The history of this strain of Mycobacterium bovis has been reviewed by Irvine (1949) and Rosenthal (1957). The organism was isolated by Nocard in 1902 from the udder of a tuberculous cow.

Calmette had established a laboratory for tuberculosis research at Lille in 1894 as a branch of the Pasteur Institute. Guèrin joined the laboratory in 1897. While repeatedly culturing the Nocard strain of M. bovis on glycerine-bile-potato medium, Calmette and Guèrin found the bacillus had lost its virulence, first for calves , then for monkeys, guinea pigs, rabbits, horses and cattle. This attenuated strain of M. bovis has been known as BCG since 1921 (Rosenthal, 1957).

The first attempt to use BCG prophylactically in man was undertaken by Weill-Hallé (1921). It was administered orally at three, five and seven days after birth to an infant born of a tuberculous mother and nursed by a tuberculous

grandmother. The child did not develop tuberculosis. As a result, routine vaccination with BCG was initiated at the Charité Hospital in Paris.

In 1928, at a technical conference to evaluate vaccination against tuberculosis with BCG, it was reported that BCG was a harmless product and an effective vaccine if administered orally to 10-day-old infants (League of Nations, 1928).

The tragedy at Lübeck in 1930 is well known (Rosenthal, 1957). The vaccine was administered orally to 240 children. Seventy-two died of tuberculosis. It was later established that the vaccine was contaminated with the Kiel strain, a more virulent organism. Since then, there has been no universal agreement on the innocuousness or efficacy of BCG.

The route of inoculation has also been open to question. Calmette et al. (1933) detected BCG in an infant's blood three to five hours after the oral administration of a single dose of 30 mg. Similar results were obtained from two chimpanzees which had received one gram of the vaccine. Subsequently, the oral route was used in South America, Canada, Rumania and Scandinavia, and later in New York by Kereszturi and Park (1936) and Levine, et al. (1948). The general fear was overcome after de Assis (1945) successfully administered a dose as large as 60 mg to infants without production of disease. Encouraged by his initial success, de Assis (1947), within four months, used 100 mg as a single dose for each of 15,000 infants. Over 85 per cent of them

became tuberculin positive. The reaction to tuberculin was used as a measure of tuberculoimmunity.

Martins and Sampaio (1952) adopted de Assis' method and within a year vaccinated 152,640 persons which included 81,607 infants. Rosemberg (1952) adopted the "concurrent or concomittant" method by which infants who lived in tuberculosis contaminated environments were frequently revaccinated. He recommended this method for tuberculin positive as well as tuberculin negative subjects.

The first study of the persistence of tuberculin sensitivity after intradermal injections of BCG was undertaken by Anderson and Belfrage (1939). At the Gothenburg tuberculosis dispensary, 905 BCG vaccinated children and adults were tuberculin tested during an 11 year period. Three hundred and ninety seven of them resided with tuberculin positive individuals. Three hundred and one had not been exposed to tuberculosis while the remaining 207 had probably been in contact with virulent bacilli. Tuberculin sensitivity was about the same in each group. Wasz-Höckert (1948) performed tuberculin tests on 1702 children from six months to seven years after the administration of BCG. Between six and twelve months, 4.1 per cent were negative, 7.5 per cent after one year, 9 per cent after two years, 9.3 per cent after three years, 11.3 per cent after four years, 14.9 per cent after five years, 17.5 per cent after six years and 9.7 per cent in the seventh year. Enell (1952) found that approximately 90 per cent of 4,000 vaccinated

school children remained sensitive to tuberculin for seven to eight years.

Rosenthal developed a multiple puncture modification of the subcutaneous method of administration which provided an appreciable amount of protection and was reportedly safer than the other methods (Rosenthal, 1939; Birkhaug, 1944, 1949; Frappier and Denis, 1945; Frappier, et al., 1952; Mande and Hüet, 1952; Hall and Wylie, 1952). The greatest sensitivity to tuberculin was produced by a vaccination using 40 punctures (Birkhaug, 1949). There is no significant difference, however, in the immunity afforded by the various methods (Federal Security Agency, U. S. Public Health Service, 1949; Edwards, et al., 1953; British Medical Research Council, 1959).

As a result of a single or multiple-puncture intradermal vaccination with BCG, primary tuberculosis in all age groups and tuberculous meningitis in children under a year has been reduced by 70 to 80 per cent (Dahlström, 1933; Hyge, 1947; Aronson, 1948; Heimbeck, 1948; Ferguson and Simes, 1949; Dahlström and Difs, 1951). For example, the study of Dahlström and Difs from 1941 to 1947 involved 20-year-old Swedish soldiers of comparable race, housing and diet. The vaccinated group of 36,235 soldiers and the unvaccinated control group of 25,239 were examined periodically by X-ray. Tuberculosis developed in the vaccinated group and in the control group at a 1:3.5 ratio.

Vaccination with BCG affords some protection (Shaw and

Palmer, 1955; Rosenthal, 1955, 1957; Rosenthal and Leppmann, 1953; British Medical Research Council, 1956, 1959). The British Medical Research Council study of 14-year-old urban school children in England demonstrated that BCG vaccination reduced tuberculosis by 82 per cent in a five year period. They stated in conclusion: "According to the present results, if none of the tuberculin-negative entrants had been vaccinated, 165 cases of tuberculosis would have been expected among them within thirty months of entry. If all of them had received BCG vaccine, 30 cases would have been expected. The difference of 135 cases represents a reduction of 82 per cent in the incidence of tuberculosis in the tuberculin-negative group." A vole-bacillus (M. microti) vaccine was used in the study also and compared favorably with BCG.

Numerous workers have reported the dissociation of the tubercle bacilli, including BCG, but the use of S and R to denote the colony forms has been controversial (Petroff, 1927; Petroff, et al., 1929; Petroff and Steenken, 1930; Steenken, et al., 1934). Steenken (1935) suggested a new terminology which is accepted as standard by the Trudeau Laboratory. The standard S and R are modified by sub letters; for example, R_V = rough virulent, R_a = rough avirulent, etc. The relatively avirulent strain of BCG R_1 has been used in experimental animals over fifty years. It can dissociate into R_{1V} which produces progressive disease in guinea pigs with silicosis and R_{1a} which is avirulent. (Steenken and Gardner, 1946). The strain of BCG_a used for

vaccination of man which is less virulent than R_1, may dissociate to R_1 . Because of this, and because the administration of BCG may give rise to an accelerated type of local reaction in individuals sensitive to tuberculin, the American Trudeau Society (1948) recommended that only tuberculin negative individuals be vaccinated.

When one mg BCG is injected intradermally, a local abscess accompanied by fever results after three weeks. A dose of 0.05 mg is said to be the optimum amount for a single dose vaccine (Wallgren, 1928). Tubercles are produced in many tissues if BCG is injected intravenously. If intradermal or subcutaneous inoculations are made, the lesions are localized at the site of injection. Bacilli may be widely disseminated but no microscopic or macroscopic lesions are found in internal organs.

The untoward reaction in man after vaccination with BCG, the possibility of reversal to a virulent organism, death of organisms during storage, the difficulty of standardization, and the possibility of contamination with more virulent bacilli, are the basis for the contention that the risks outweigh the usefulness of BCG as a vaccine (Weiss, 1959). In spite of the lack of agreement, Denmark, Norway, France, Brazil and Japan legally enforce vaccination with BCG. It is estimated that approximately 17 million people have been vaccinated (Rosenthal, 1957).

Calmette and Guèrin (1927) reported that cattle were afforded protection by the annual intravenous or subcutaneous

injections of BCG. Watson et al., (1928) vaccinated 17 calves four to seven days old with 75 to 100 mg BCG. The calves were fed milk from tuberculin positive herds until six months of age and subsequently pastured and stabled with tuberculin positive cattle. No signs of tuberculosis were detectable during the following two years. When the animals were slaughtered, tubercles were found in many of the organs by gross examination. It was concluded that an improper dose of vaccine had been administered.

Larson and Evans (1929) inoculated cattle with living and heat inactivated BCG. Six months after vaccination, the animals were housed with tuberculin positive cattle. When the animals were slaughtered, 88.8 per cent inoculated with living BCG, 44.4 per cent inoculated with killed BCG, and 87 per cent of the controls were tuberculous. Vaccination with living BCG had afforded no protection.

Buxton and Griffith (1931) and Buxton, et al.(1939) substantiated the findings of Calmette and Guèrin (1927). Cattle which had previously received BCG intravenously survived eight months to three years after inoculations with virulent organisms. Unvaccinated animals survived inoculations with virulent organisms less than 60 days.

Swine, at 5 and 20 days of age, were vaccinated subcutaneously with 10 to 40 mg BCG (Jundell and Magnusson, 1935). The tuberculoimmunity of the animals was challenged with virulent bacilli 13 weeks after the last vaccination. There was no evidence of disease in the pigs vaccinated

subcutaneously five to nine weeks after the challenge inoculation. Repeated subcutaneous vaccination with BCG increased porcine resistance to tuberculosis.

Guinea pigs are highly susceptible to virulent M. bovis and M. tuberculosis. Increased resistance in guinea pigs by BCG has been demonstrated by many workers (Jensen, 1930; Bogen and Loomis, 1935; Schwabacher and Wilson, 1938; Liebow, et al., 1940). Bogen and Loomis vaccinated more than 325 guinea pigs during a five year period. They found a marked reduction in the number of animals that were tuberculous as compared to control animals. In a study by Seagle, Karlson and Feldman (1953), 54.5 per cent of control animals died and none of the vaccinated guinea pigs had died 166 days after the inoculation of virulent bacilli.

Schwabacher and Wilson (1937) noted that there was a statistically significant difference between vaccinated and control mice which survived inoculation of virulent organisms when the vaccinated animals had received $2x10^7$ living BCG organisms. Vaccinated animals survived 125 days. The unvaccinated animals survived for 72 days. Swedberg (1951) found that the unvaccinated mice died within 19 days and vaccinated mice died 44 days after the inoculation of virulent organisms. Different strains of mice vary markedly in their natural resistance to experimental infection (Youmans, et al., 1959).

Lurie (1936) demonstrated that Mycobacterium bovis was inhibited or destroyed in rabbits vaccinated with BCG.

Bacilli suspended in India ink or trypan blue solution were mixed with 6 per cent melted agar and injected subcutaneously into normal and BCG-vaccinated rabbits. At various subsequent intervals, there were fewer bacilli in BCG-vaccinated rabbits than in the unvaccinated rabbits as determined by plate counts of the bacilli from the agar.

Lurie (1942) collected monocytes from normal and BCG-vaccinated rabbits. After allowing them to phagocytize virulent tubercle bacilli *in vitro* in the presence of serum obtained from normal rabbits and rabbits vaccinated with BCG, these mixtures were introduced into the anterior chamber of the eye of the normal rabbits. The proliferation of the virulent bacilli was determined by plate counts and microscopic technique. Fewer virulent bacilli were detected in the monocytes of vaccinated rabbits than in monocytes of unvaccinated rabbits. This was attributed to the increased ability of mononuclear phagocytes of BCG-vaccinated rabbits to ingest the bacilli. The increased phagocytosis was non-specific. Staphylococci, carbon particles and collodion were also phagocytized more readily.

Virulent bacilli are not immediately destroyed in BCG-vaccinated animals. The protection arises from the inhibition of bacillary multiplication (Levy, *et al.*, 1961). Elberg (1960) postulates that the tubercle bacillus resides within the monocytes of BCG-vaccinated rabbits as an "elementary particle". The monocytes prevent the proliferation of virulent bacilli within them. The site of the

tuberculoimmunity is cellular. Elberg has compared the cellular tuberculoimmunity to lysogeny in those bacteria rendered immune to infection by homologous viral particles.

Cellular resistance has been transferred passively by histiocytes and lymphocytes (Fong, et al., 1962). Cells were extracted from the peritoneal exudates of BCG-vaccinated rabbits and whole cells or cell lysates were injected intradermally into unvaccinated rabbits. Cells were removed 13 days later from the peritoneal cavities of the recipient rabbits and exposed to virulent M. tuberculosis in vitro. The cells survived 25 to 42 days. Approximately 47 per cent less of the control cells were living on comparable days.

Tuberculoimmunity afforded by bacterins prepared with phenol. Bacterins have been prepared from the various species of mycobacteria with physical and chemical agents. Heat, ultra-violet irradiation, sunlight, urea, oleic acid, iodine, fluorides, chlorine, toluene, petroleum, ether, lecithin, phenol, formalin, glycerine, hydrochloric acid, sodium hydroxide, nitrous acid, ethylene oxide and prolonged storage have been used (Weiss, 1959).

Bacterins prepared with phenol have been widely investigated. The degree of tuberculoimmunity conferred is controversial.

Trudeau (1894) reported that rabbits vaccinated with phenol-inactivated M. avium were not afforded tuberculoimmunity. In 1936, Ferguson and Cannon administered 0.1 mg

of 5 per cent phenol-inactivated M. tuberculosis to 137 guinea pigs intratracheally. The tuberculoimmunity of the animals was challenged four weeks later with virulent bacilli by the same route. The average survival time was twice as long for the vaccinated animals as for the control group. When all the animals were killed and examined macroscopically, tubercles were less extensive in the vaccinated guinea pigs than in the controls. Bloch and Segal (1955) and Meyer (1956), in a similar experiment, compared the protection afforded mice and guinea pigs by living BCG and virulent tubercle bacilli killed with phenol. They concluded that living BCG was consistently a superior immunizing agent.

Vahlne, et al. (1955) administered three subcutaneous injections of phenolized, hexane-extracted tubercle bacilli to guinea pigs at weekly intervals. The resistance of the animals was challenged 36 days later with virulent bacilli. A degree of protection was obtained but the experiment was discontinued after 72 days. The draining tuberculous abscesses at the site of injection of the virulent bacilli were considered too hazardous to the handlers.

Resistance of mice was increased with phenol-inactivated, acetone-washed avirulent M. tuberculosis (Weiss and Dubos, 1955). Weiss (1958) reported that phenol-inactivated bacilli were as immunogenic for guinea pigs as BCG.

Acharaya, et al. (1958) determined the relative immunity afforded mice by phenolized and living virulant bacilli as measured by the mouse corneal assay method of

Rees and Robson (1950) and Robson and Sullivan (1957). The degree of resistance was estimated by the relative number of microscopic lesions in the infected eye. Phenolized preparations, in adequately large amounts, produced immunity comparable with that elicited by BCG.

Weiss and Wells (1960) treated M. phlei and virulent M. tuberculosis with 2 per cent phenol for 24 hr . The cells were washed repeatedly with distilled water and acetone. Groups of guinea pigs were inoculated intraperitoneally, subcutaneously or intramuscularly with 1.5 mg to 24.0 mg dry weight cells suspended in saline or Freund's adjuvant. Another group of guinea pigs was vaccinated with 3×10^6 cells of BCG. One group of guinea pigs was inoculated with saline as control animals. The tuberculoimmunity of the animals was challenged at 6, 12 or 36 weeks intramuscularly or by aerosol with virulent M. tuberculosis. A total of 330 guinea pigs was used in the study. The criterion was the mean survival time. Mycobacterium phlei was devoid of any detectable immunogenic activity, only M. tuberculosis induced an increased resistance. The optimum dose of phenolized cells in saline for mice and guinea pigs was 1 to 1.5 mg administered intraperitoneally. If the killed bacilli were suspended in Freund's adjuvant, 1.0 to 3.0 mg injected subcutaneously proved most protective. The tuberculoimmunity was equal to that induced by BCG.

Weiss and Wells made several succinct criticisms of other studies of experimental tuberculoimmunity. If killed

organisms or extracts could be injected at a level which would be equal to the total number of organisms resulting from *in vivo* multiplication of BCG, bacterins might be as immunogenic as BCG. Moreover, the route of administration, the length of time between vaccination and challenge, the species of animal used and the nutritional level, are variables which are frequently disregarded.

Extracts of mycobacteria. Various substances obtained from mycobacterial cells are antigenic but there is no evidence that a single substance constitutes that component of the tubercle bacilli which elicits tuberculoimmunity.

Koch (1897) produced a water soluble fraction of ground tubercle bacilli. It did not immunize guinea pigs. The residue, "Tuberculin R", was both non-toxic and immunogenic.

Martin and Vandremer (1906) injected guinea pigs with defatted tubercle bacilli which protected them against experimental infections.

Dreyer (1923a, 1923b) removed the lipids from tubercle bacilli to obtain a product he named "diaplyte". Some therapeutic value was credited to diaplyte and large doses did not produce ulceration at the site of injection. Kettle (1924), Douglas, *et al.*, (1925) and Grasset (1925) reported that "diaplyte" possessed no therapeutic value. Brofenbrenner and Straub (1925) found that "diaplyte" hastened the death of tuberculous guinea pigs.

Dreyer, Vollum and Amitzböll (1934) administered

"diaplyte" prophylactically for nine years in a herd of cattle. At the beginning of the experiment approximately 90 per cent of the cattle were tuberculin positive. Calves were given one intraperitoneal or intravenous inoculation with 175 mg dry weight of "diaplyte" and at the end of the ninth year, only 11 per cent of the animals were tuberculin positive.

Aqueous bacillary extracts were more immunogenic than lipoidal fractions (Widstron, 1941). Carbohydrate-lipid fractions of a virulent strain of M. tuberculosis possessed prophylactic and therapeutic values if injected subcutaneously into guinea pigs (Kropp and Floyd, 1947). Seibert (1950) reported that purified tubercle protein and polysaccharide fractions were non-immunizing. Anderson's (1927) phosphatide fraction of tubercle bacilli did not immunize rabbits (Kubo, et al., 1951). Boquet and Nègre (1923), Nègre and Boquet (1930) and Negre (1952, 1956) prepared a heat-killed, acetone washed, methanol-soluble extract of tubercle bacilli, Antigène Méthylique. It contained phosphatides primarily and possessed some therapeutic and prophylactic value for guinea pigs and rabbits.

Choucroun (1943, 1947, 1949, 1956) reported that a fraction of tubercle bacillus extracted with paraffin oil was immunogenic for guinea pigs. This was composed principally of one of the waxes, Wax D (Crowle, 1958a).

The most promising of the various tubercle bacillary extracts are those of Youmans, et al. (1957), Youmans, et

al. (1959), Kanai and Youmans (1960), Kanai, et al. (1960). The fraction induced tuberculoimmunity in guinea pigs and mice. It was extracted from avirulent M. tuberculosis suspended in phosphate buffer-isotonic sucrose solution and disrupted in a ball mill with powdered glass at 4 C. Any intact cells were removed by centrifugation at low speed. The supernatant fluid was centrifuged at increasing speeds in a Spinco Model L ultracentrifuge. All sediments obtained by centrifuging at less than 144,700 X g were discarded. The final product was the sediment from three hrs centrifugation at 144,700 X g. It was enzymatically active, mitochondria-like, tuberculoimmunogenic for mice, and reddish in color. Hence, the extract was named "red fraction". In 1960, these investigators disrupted the cells with the Pressure Cell Method of Ribi, et al. (1958). The immunogenic fraction was not red, and therefore, the name "red fraction" was no longer applicable (Kanai, et al., 1960). The particles were large enough to be studied by electron microscopy. They were composed of ribonucleoprotein and fragments of cytoplasmic membrane. They were not mitochondria-like. An injection of 10 mg of the extract intraperitoneally into CF-1 and Strong A strains of mice was the optimum dose to afford protection four weeks later from virulent bacilli injected intravenously. The criterion was the difference in the per cent of vaccinated and unvaccinated mice which survived 30 days after challenge. No tuberculin sensitivity was induced by the extract. The

immunogenicity of the intracellular particles was superior to that of cell wall fraction and intact avirulent M. tuberculosis. The cell wall fraction had no immunogenic activity but induced tuberculin hypersensitivity. The component responsible for the induction of hypersensitivity was not destroyed by trypsin, pepsin or ribonuclease but was removed by treatment with alkaline alcohol.

The mechanism by which the particles elicit tuberculoimmunity has not been determined. The particles are rich in enzymes and are principally ribonucleoproteins. Since ribonucleoproteins occur in the ribosomes and are associated with protein synthesis (Allfrey and Mirsky, 1961), the particles may enter into the production of proteins, enzymes or others. They may be directly or indirectly responsible for the production or action of some constituent which is adverse to mycobacterial survival and growth.

The immunogenicity of the intracellular particles was destroyed by dilution with distilled water, exposure to 60 C for 30 minutes, by extraction with acetone or ether, by electron bombardment and sonic vibration. The suspending medium during extraction of the particles is critical. Cells are suspended in buffered isotonic sucrose, 0.25 mg, although 0.88 M sucrose is equally effective in preserving the integrity of the mitochondria.

There have been no substantiating reports of the immunogenicity of the extract prepared by Youmans' method for mice, or its activity in other animals.

An extensive review of the studies of the various fractions of the tubercle bacillus and their immunogenicity was made by Crowle (1958a). Crude tuberculo-polysaccharides as well as a purified high molecular weight polysaccharide as prepared by Siebert (1950) are antigenic but not immunogenic. Tuberculoproteins from bacillary culture medium or from tubercle bacilli, alone or bound to polysaccharide elicit production of specific antibodies which are responsible for an immediate type of hypersensitivity. A mixture of the proteins and waxes induces the delayed type of hypersensitivity.

Lipids are responsible for the majority of the physical and chemical properties of the mycobacteria. For example, tuberculolipids are required to produce delayed hypersensitivity, are associated with the acid-fastness, hydrophobic nature and virulence of the tubercle bacillus, and with antibody production and tuberculoimmunity. The lipids may be readily extracted from the cells and are classified as acetone-soluble fats, phosphatides, waxes and "firmly bound" lipids. With the exception of the "firmly bound" lipids, the lipids are extracted from intact mycobacteria with neutral organic solvents. Acetone soluble lipids do not immunize guinea pigs, rabbits or mice. They are toxic and increase the susceptibility of these animals to the tubercle bacillus. It is recommended that bacterins be washed with acetone before they are injected into experimental animals (Crowle, 1958a).

Phosphatides may be extracted from bacilli with ether-alcohol, some of which are toxic for experimental animals. Antigène méthylique, a methanol extracted phosphatide which contains a small amount of fats, waxes and complex nitrogenous compounds, possesses some prophylactic and therapeutic properties (Crowle, 1958a). It elicits the production of antibodies specific for the phosphatides. However, if the phosphatide is carefully purified, it is an incomplete antigen, a complex haptene. The tuberculoimmunity conferred by Antigène méthylique to experimental animals differs from that by vaccines or bacterins. The acquired resistance is present within two weeks after inoculations, no tuberculin sensitivity develops, and the resistance is relatively short-lived. The therapeutic value of the extract and its ability to enhance BCG vaccinations differentiates its activities from the bacterins and vaccines.

Natural or increased resistance to tuberculosis in animals is correlated with a high phosphatidase activity of the tissues. Vaccination with phosphatide extracts or a synthetic phosphatide stimulates enzyme production which is correlated with the increased resistance. This is strong evidence that tuberculoimmunity is not entirely, if at all, associated with the presence of a circulating specific antibody.

The waxes are separated into A, B, C, and D by differences in their solubilities. Wax A contains esters of phthiocerol with fatty acids, and a free fatty acid, mycolic

acid. Wax A sensitizes guinea pigs but does not immunize them against virulent M. bovis and M. tuberculosis. Wax B has been studied very little. Wax C and Wax D confer no tuberculoimmunity. Wax D is a polysaccharide ester of mycolic acid and contains nitrogen and phosphorus. Another wax, PMKO, is extracted from intact tubercle bacilli with warm paraffin oil. It is probably identical to Wax D and like it, possesses no immunogenic properties (Crowle, 1958a).

Specificity of tuberculoimmunity. The specificity of classical tuberculoimmunity is less than that of many well known humoral antibody immunities. For example, the immunity produced by a given type of Diplococcus pneumoniae does not immunize against another type. This is not true of the mycobacteria since cross immunity and sensitivity may occur with M. bovis, M. tuberculosis, M. balnei, M. ulcerans and M. phlei (Crowle, 1958a) and taxonomically unrelated bacteria induce some degree of tuberculoimmunity. Nukada and Ryu (1936) demonstrated that a mixture of Salmonella typhosa and Neisseria gonorrhoea injected into pigs or mice induced some increased resistance to tuberculosis. A vaccine of Bordetella pertussis also protected mice when subsequently inoculated with M. tuberculosis (Dubos and Schaedler, 1956).

If mice were previously infected with virulent or avirulent Brucella abortus and subsequently inoculated with M. tuberculosis they were as resistant as other mice vaccinated with BCG (Nyka, 1956). This resistance differed

from that elicited by BCG in that BCG immunized equally well by the intravenous, intraperitoneal or subcutaneous routes. Brucellae were effective only when injected by the intravenous route. Nyka (1956) proposed that the brucellae induced antitubercular resistance because of the common route of infection. The brucellae conditioned the cells to resist a subsequent exposure to virulent mycobacteria.

Evaluation of tuberculoimmunity. The evaluation of the tuberculoimmunity afforded animals by vaccination may be influenced by diet (Dubos and Pierce, 1948; Dubos, 1955), the strain of animal (Youmans, 1957), the dose and kind of vaccine or bacterin, the amount and route of administration of the challenge dose (Raffel, 1960), and the criterion chosen for the demonstration of tuberculoimmunity (Youmans, 1957). These problems have been analyzed effectively by Crowle (1958a) who has stated:

> "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 against any disease can be defeated by severe enough infection. 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 LD_{100} 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."

In experimental tuberculoimmunity, laboratory animals are vaccinated with BCG or bacterins prepared from the various mycobacteria (Youmans, 1957). At some subsequent time varying from three weeks to three months (Bloch and Segal, 1955), their resistance is challenged intravenously, intraperitoneally or by aerosol with virulent M. tuberculosis or M. bovis (Weiss and Wells, 1960). Some time after challenge, vaccinated and control animals may be killed and the relative number of microscopic and macroscopic lesions in the organs and tissues recorded (Feldman, 1943; Youmans, 1949; Larson and Wicht, 1962). It is presumed that immunized animals should have less number of tubercles than unvaccinated controls. This method is very subjective and is not reliable (Youmans, 1957). Moreover, previously sensitized animals have more extensive lesions initially than the controls. These lesions tend to diminish with time (Opie and Freund, 1937).

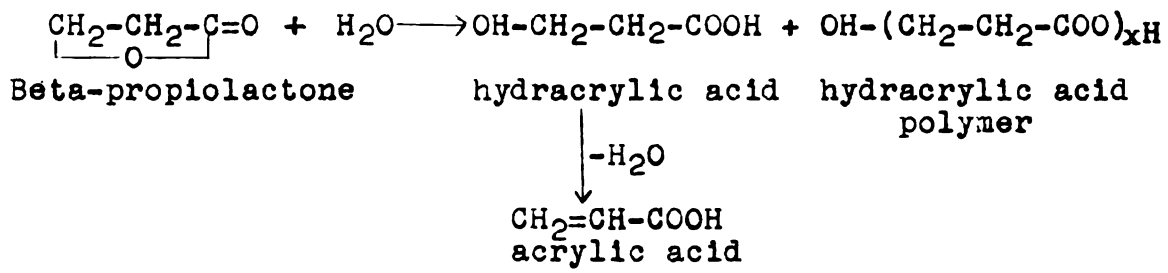
The comparative number of acid fast bacilli which are isolated from the organs and tissues of vaccinated and control animals has been used as another index of immunity (Crowle, 1958a; Panisset and Benoit, 1959; Dubos and Conge, 1960). Its validity is questionable because the reduction

in the number of virulent mycobacteria in the organs of vaccinated animals requires a considerable period of time (Youmans, 1957).

The most frequently used method of evaluation of immunity is the difference in the mean or median days control and vaccinated animals survive following challenge with virulent bacilli. It may be reliable if the deaths of vaccinated and control animals are normally distributed but Youmans (1957) has demonstrated that the distribution of death of vaccinated mice following challenge with virulent M. tuberculosis was not normal whereas that of unvaccinated mice was normal. The only method of evaluation recommended by Youmans (1960) was the comparison of the number of vaccinated and unvaccinated animals surviving at a specific time interval after the inoculation of virulent bacilli. The 30th day was the end point most frequently chosen.

The results obtained may be equivocal. Bloch and Segal (1955) found that BCG-vaccinated mice did not survive as long as unvaccinated control mice. This was interpreted as due to the more extensive lesions usually associated with previous sensitization in vaccinated animals.

Beta-propiolactone. Beta-propiolactone (BPL) in the concentrated state is a colorless liquid, stable at low temperatures, but quickly hydrolyzed in aqueous solutions into an inactive, non-toxic acid, hydracrylic acid. This acid is removed more quickly in plasma than in water (Testagar and Co.). The formulas are as follows:



Beta-propiolactone polymerizes at high temperatures to hydracrylic acid polymers.

It has a boiling point of 51 C at 100 mm Hg, a specific gravity of 1.1490, a refractive index of 1.4131, a melting point of -33.4 C, a flash point (open cup) at 74 C and a half life hydrolysis in water of three to four hours (Wilmot, 1959).

At ordinary temperatures, BPL is miscible with most organic solvents and reacts readily with hydroxyl, amino, carboxyl, sulfhydryl and phenolic groups (Gresham, et al., 1948). Beta-propiolactone is irritating to the eyes and mucous membranes of the respiratory tract.

Beta-propiolactone has been used to sterilize plasma (LoGrippo, 1960), inactivate viruses (LoGrippo and Hartman, 1955; Mack and Chtisen, 1956), certain bacteria, bacterial spores and fungi (Wilmot, 1959). Huddleson (1955) found that the concentrations required for sterilization varied with different organisms, the time of exposure, and the medium in which the cells were suspended.

Yerian and Teodoso (1961) investigated the nature of the antibacterial action of BPL. They studied the cytological changes, viability and respiratory activities of Escherichia coli. No correlation was found to exist between

the loss of viability and any morphologic alteration. They demonstrated by the Warburg technique that BPL acted as an inhibitor of respiration. As little as 0.05 per cent BPL inactivated E. coli (Yerian and Teodoso, 1961) and 0.2 per cent inactivated viruses (LoGrippo, 1960) but at least 5 per cent BPL was required to detoxify staphylococcal enterotoxin (Hopper and DeValois, 1961).

Bruch (1961) used BPL vapor to decontaminate certain enclosed spaces in which spore samples on paper strips were placed. The length of time required to inactivate the dry bacterial spores with the BPL vapor varied with temperature, relative humidity and concentration of the vapor. Beta-propiolactone vapor possessed poor penetrating power.

Curran and Evans (1956) killed the spores of Bacillus subtilis, B. stearothermophilus, B. cereus and Clostridium sporogenes in two hours with 0.3 per cent BPL. Bacillus globigii spores required 0.75 per cent BPL for one hour or 0.5 per cent for two hours (Testagar and Co.). Continued incubations beyond two hours did not increase the inactivation (Curran and Evans, 1956).

LoGrippo (1960) used BPL to sterilize plasma and tissue grafts and treat viral suspensions for vaccines. The pH of the viral suspension during the period of inactivation was maintained at 6.8 to 7.4. Complete inactivation of virus in 50 ml amounts occurred with 0.2 per cent BPL. However, if seven liter suspensions were treated with a final concentration of 0.3 per cent ot 0.4 per cent of BPL, all viral

particles were not inactivated.

A desirable inactivating agent for the preparation of bacterins should possess the following properties: 1) lethal for microorganisms, 2) low toxicity for other living cells, 3) a good preservative, 4) no allergenic properties, and 5) a margin of safety between the killing dose and the toxic dose. Beta-propiolactone possesses all these properties (LoGrippo, 1960). Moreover, in the concentrations used for inactivation, BPL does not denature proteins and preserves the antigenic integrity of the microorganisms. For these reasons, the ability of BPL to inactivate M. bovis for use as a bacterin appeared worthy of investigation.

MATERIALS AND METHODS

Glassware. All glassware was soaked for 30 min in an alkaline detergent (Wyandotte Chemical Corp.[1]) containing no wetting agent. After washing in the detergent, glassware was rinsed 2 times in tap water and 3 times in distilled water.

Media. Dubos Agar and Dubos broth with enrichment (Difco[2]) were the media used for cultivation of the mycobacteria.

Cultures. A laboratory strain of Mycobacterium bovis (Ravenel)[3] and BCG[3] were used as the source of organisms to prepare the bacterins and vaccine.

Animals. Experimental animals were four-week-old male and female albino Swiss mice[4] and female and male albino guinea pigs (450 g to 820 g)[5].

Preliminary studies on the inactivation of M. bovis (Ravenel) by beta-propiolactone. Beta-propiolactone (BPL)[6] was stored in a polyethylene container in the refrigerator at 4 C. It was diluted immediately prior to use.

For determination of the inactivation of M. bovis by BPL, cultures were grown 14 days in broth. They were centrifuged 20 min at 1680 X g and the supernatant fluid removed.

1. Wyandotte Chemical Corp., Wyandotte, Michigan.
2. Difco Laboratory, Detroit, Michigan.
3. Tuberculosis Unit, Communicable Disease Center, Atlanta, Ga. (original cultures).
4. Rockland Farms, New York City, N. Y.
5. Breeding stock obtained from Biol. Lab., Camp Detrick, Maryland.
6. Wilmot Castle Company, Rochester, New York.

The sediments were pooled, resuspended in 6 ml sterile triple glass distilled water to approximately 1 mg wet weight per ml and redistributed in equal volumes into six centrifuge tubes. The tubes were placed in an ice bath and the pH of the contents adjusted to approximately 8.4 with 0.5 M $Na_2HPO_4 \cdot 12\ H_2O$. Nine ml each of 0.00001, 0.0001, 0.001, 0.01 and 0.1 per cent BPL were prepared in ice-cold sterile triple distilled water and added to five tubes of the bacterial suspensions. Sterile triple glass distilled water was added to the sixth tube for the control. The contents were mixed by agitating the tubes by hand and then were placed in a water bath at 37 C. Ten minutes were allowed for the contents to reach thermal equilibrium. The suspensions were incubated for 2 hr during which time the pH was maintained at 7.6 ± 0.4. After incubation, the suspensions were centrifuged at 1680 X g for 20 min in an International centrifuge, Universal Model UV. The supernatant fluid was removed and the sediments were suspended in 1 ml of sterile distilled water. Ten-fold dilutions and drop plates (Mallmann and Peabody, 1962) were made of the suspensions. After incubation, the number of colonies was counted and recorded.

To determine the effect of pH and the time of treatment on the inactivation of M. bovis by BPL, approximately 10 mg wet weight per ml of M. bovis were suspended in 0.4 per cent BPL in three separate tubes. The first tube was incubated for 2 hr at 37 C and the pH adjusted at 15 min intervals to

pH 7.6 ± 0.4. The second tube was maintained at the same pH but incubated for 1 hr and the third tube was incubated for two hr without adjustment of the pH. A control tube contained a comparable number of cells suspended in 2 ml of sterile glass distilled water and incubated at 37 C for 2 hr at pH 7.6 ± 0.4. Drop plates were made and incubated at 35 C.

To determine if the efficiency of BPL were influenced by cell concentration, approximately 15 mg wet weight of cells per ml of distilled water were dispensed in each of seven centrifuge tubes, centrifuged, and the supernatant fluid removed. After adjusting the pH to 8.4, BPL was added to the cells in respective tubes to yield a final concentration of 0.001, 0.01, 0.1, 0.2, 0.3, and 0.4 per cent. Triple glass distilled water was added to the cells in the seventh tube as a control. The tubes were incubated 2 hr at 37 C.

Preparation of bacterins. BPL-inactivated M. bovis: Three-week-old broth cultures were centrifuged 20 min at 1680 X g and the cells resuspended in triple distilled water to approximately 10 mg wet weight per ml. The tubes were placed in ice-cold water and the contents were adjusted to pH 8.4. With constant agitation, BPL was added to make a final concentration of 0.4 per cent. After thermal equilibration in a water bath, tubes were incubated at 37 C for 2 hr during which time the tubes were shaken and the contents adjusted to pH 7.6 ± 0.4 at 15 min intervals.

After incubation, the cells were centrifuged at 1680 X g for 20 min and washed 4 times with sterile distilled

water. The cells were standardized as indicated below and used as BPL-bacterin.

Phenol-inactivated M. bovis: Viable M. bovis cells were washed as above and resuspended in a 2 per cent phenol solution. The suspension was incubated at room temperature for 24 hr. It was shaken intermittently during incubation. The treated cells were washed 4 times with triple distilled water, standardized as noted below and used as phenol-bacterin.

Acetone washed bacterins: Portions of BPL-bacterin and phenol-bacterin were washed once with sterile distilled water and 3 times with cold acetone. The cells were standardized as noted below and used as BPL-acetone-bacterin and phenol-acetone-bacterin.

Standardization of bacterins. The bacterins were standardized on the basis of mg wet weight per ml, using the Hopkins Tube Method (Kubica, 1960).

Extract of M. bovis. A modification of the method of Kanai and Youmans (1960) was used to extract intracellular particles from M. bovis. Cultures were grown in Dubos broth for 23 days at 35 C. The cells were centrifuged and washed once with sterile phosphate buffer, pH 7.0. The harvested cells were suspended in 150 ml sterile sucrose buffer (0.25 M sucrose dissolved in phosphate buffer) and transferred to a Waring blendor containing approximately 120 g sterile glass beads. The blendor had previously been thoroughly polished with steel wool, washed 3 times,

rinsed repeatedly in tap water and 4 times in distilled water, and sterilized at 121 C for 30 min.

To maintain a low temperature during blending, a plastic bag was inverted over the cylinder to form a pouch into which was poured a mixture of dry ice and 15 per cent NaCl. An outside temperature of -11 C maintained an inside cylinder temperature of less than 5 C. The top of the cylinder was tightly wrapped with cheese cloth saturated with a disinfectant[1].

The Waring blendor was turned to the highest speed for 30 min, allowed to stand 40 min, and the contents transferred to 200 ml centrifuge tubes. The tubes were centrifuged in an International Centrifuge, then in a Serval Superspeed Angle Centrifuge at 4 C and the sediment discarded. The supernatant was centrifuged in a Spinco Ultracentrifuge, Model L, with Rotor Number 40 for three hr at 100,000 X g (144,700 X g at the base of the tube). The process was as follows:

Broken cells from Waring Blendor

↓

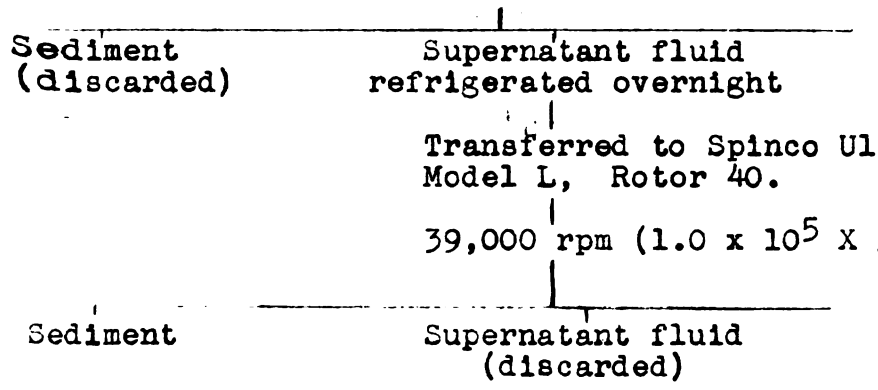
Centrifuged in International Centrifuge, Universal Model UV, 1500 rpm (427 X g) for 10 min.

↓

Increased to 3150 rpm (1885 X g) for 60 min.

Sediment (discarded)	Supernatant fluid
	↓
	Transferred to Serval Superspeed Angle Centrifuge at 4 C.
	↓
	Rheostat at 50 (11.730 X g) for 15 min
	↓
	Increased to 75 (20,360 X g) for 10 min
	↓
	Increased to 100 (36,000 X g) for 5 min

1. Septisol (ET 383), Dow Chemical Co., Midland, Michigan.



The final sediment was weighed, suspended in phosphate buffer, 100 mg per ml, and injected into experimental animals within 24 hr after preparation.

BCG vaccine. Three-week-old broth cultures of BCG were standardized on the basis of mg wet weight per ml using the Hopkin's Tube Method (Kubica, 1960).

Determination of the amount of M. bovis to be used to challenge the tuberculoimmunity of mice and guinea pigs. To determine the approximate amount of wet weight of M. bovis lethal for mice, intraperitoneal injections of 2.5, 3.5, 5.0, 6.0 and 7.2 mg of two-week-old cultures were made, two mice per each amount of inoculum. The mice were 8 weeks old, the age at which the vaccinated and unvaccinated mice were to be inoculated. The number of days after inoculation on which each of the mice died was recorded.

Guinea pigs were inoculated intraperitoneally with 1.0, 2.0 and 5.0 mg wet weight of cells, four guinea pigs per each amount of inoculum. The number of days after inoculation on which each of the guinea pigs died was recorded.

Mice and guinea pigs were examined for macroscopic

lesions after death.

Vaccination of experimental animals. A total of 80 mice were inoculated intraperitoneally. Group I (20 mice) received 1 mg BCG. Group II (20 mice) received 15 mg wet weight of BPL-bacterin. Group III (20 mice) received 15 mg wet weight of phenol-bacterin. Group IV (20 mice) received 8 mg extract. Group V (11 mice) were uninoculated and served as controls.

Ninety guinea pigs were divided equally into nine groups and inoculated intraperitoneally. Each group received one of the preparations as follows: 0.5 mg BCG, 10 mg wet weight BPL-bacterin, 10 mg wet weight BPL-acetone-bacterin, 10 mg wet weight phenol-bacterin, 10 mg wet weight phenol-acetone-bacterin, 5 mg extract, 10 mg extract and 25 mg extract. One group of the guinea pigs was uninoculated.

Challenge of experimental animals with virulent M. bovis. Four weeks after vaccination, all animals were inoculated intraperitoneally with viable M. bovis cells, 32 mg and 2 mg wet weight for the mice and guinea pigs, respectively. Animals were observed daily for 28 days and the number of deaths recorded.

Hypersensitivity. Two groups of four guinea pigs each were inoculated intraperitoneally with 5 mg BPL-bacterin and phenol-bacterin. Five weeks later each animal received intradermally 0.1 ml mammalian Old Tuberculin[1] diluted 1:20 in saline. The diameter of induration at the site of

1. Supplied by Animal Disease Eradication Division, USDA.

injection was observed at 48 hr and recorded.

RESULTS

Preliminary studies of the inactivation of M. bovis with BPL. When approximately 10 mg wet weight of cells per ml were treated with BPL at pH. 7.6 ± 0.4 for 2 hr, 0.01 per cent concentration or less of BPL was insufficient to inactivate all cells. All cells were inactivated by 0.1 per cent or more BPL. The number of viable cells per ml as determined by the Drop Plate Method (Mallmann and Peabody, 1962) was 2.7×10^8 prior to the inactivation process, 3.4×10^7 in the control culture which underwent incubation, centrifugation and washings without BPL, approximately 3.0×10^7 when a final concentration of 0.01 per cent or less BPL was used, and zero if 0.1 per cent or more was used.

A greater concentration of the cells required a greater concentration of BPL to inactivate all cells. As the previous results indicated, 0.1 per cent BPL inactivated all of 3×10^7 organisms per ml when incubated 2 hr at pH 7.6 ± 0.4. However, if 1.3×10^{12} organisms per ml were treated with a final concentration of 0.1 per cent BPL, 3.1×10^3 cells per ml were not inactivated. It required 0.2 per cent BPL to inactivate all cells if there were originally 1.3×10^{12} cells per ml.

Complete inactivation of all cells was not accomplished with 0.4 per cent BPL if 0.5 M disodium phosphate solution was not added to the suspensions to maintain the pH at 7.6 ± 0.4 during the 2 hr incubation at 37 C. The viable cell counts as determined by the Drop Plate Method were 1.8×10^{11}

per ml in the control culture, zero in the culture in which the pH was maintained at 7.6 ± 0.4, and 6.0 x 10^2 in the culture in which the pH was not adjusted. In the latter, the pH was approximately 5.

All organisms were inactivated when 5.6 x 10^{10} organisms per ml were treated with a final concentration of 0.4 per cent BPL at 37 C at pH 7.6 ± 0.4 for 2 hr. If the period of incubation was reduced to 1 hr, 2.7 x 10^3 organisms per ml remained viable.

If BPL-inactivated organisms were washed only once with distilled water, sufficient BPL or degradation products were present in 15 mg wet weight of cells to elicit signs of toxicity in mice inoculated intraperitoneally. If the cells were washed 4 or more times all detectable toxicity was removed.

Determination of the amount of M. bovis required to cause death of mice and guinea pigs. The average number of days that mice survived after intraperitoneal injection of 2.5, 3.5, 5.0, 6.0, and 7.2 mg wet weight of M. bovis was 75.5, 27.5, 14.5, 12.5 and 5.5, respectively (Table 1). Gross lesions in the liver, spleen or kidney were present in only those mice which lived 22 days or more after inoculation.

The average number of days that guinea pigs survived after intraperitoneal injections of 1.0, 2.0 and 5.0 mg wet weight M. bovis was 31, 19 and 8.5 respectively (Table 2). Gross lesions in the liver, spleen or lung were present in

TABLE 1. The number of days eight-week-old mice survived after intraperitoneal injections of Mycobacterium bovis.

Mg inoculum per mouse	Number of days each mouse* survived post-inoculation	Mean of days survived
2.5	76** 75	75.5
3.5	22** 33**	27.5
5.0	19 10	14.5
6.0	15 10	12.5
7.2	7 4	5.5

* 2 mice per inoculum
** Gross lesions observed in liver, spleen and/or kidney.

TABLE 2. The number of days guinea pigs survived after intraperitoneal injections of Mycobacterium bovis.

Mg wet weight inoculum per guinea pig	Number of days each guinea pig * survived post-inoculation	Average number of days survived
1.0	25**	
	28**	
	32**	31
	39**	
2.0	15	
	16	
	19	19
	26**	
5.0	6	
	8	
	10	8.5
	10	

* 4 guinea pigs per inoculum
** Gross lesions observed in liver, spleen and lungs

only those guinea pigs which lived more than 19 days after inoculations.

Survival of vaccinated and unvaccinated mice after inoculation with M. bovis. When unvaccinated control mice and mice vaccinated 28 days previously were inoculated intraperitoneally with 32 mg wet weight of M. bovis, less than one half the unvaccinated (control) mice were living on the 6th day after inoculation (Table 3). At the same time interval after inoculation, 81 per cent of the mice which had received BPL-bacterin, 72 per cent which had received BCG, and 53 percent which had received phenol-bacterin were living (Table 4).

On the 7th day post-inoculation, 34 per cent of the control mice, 72 per cent of the BCG vaccinated mice, 69 per cent of the BPL-bacterin vaccinated mice and 58 per cent of the phenol-bacterin vaccinated mice were living.

On the 10th day post-inoculation, 34 per cent of the control mice, 72 per cent of the BCG vaccinated mice, 62 per cent of the BPL-bacterin vaccinated mice and 50 per cent of the phenol-bacterin vaccinated mice were living.

At the 24th day post-inoculation, 18 per cent of the control mice, 45 per cent of the BCG vaccinated mice, 42 per cent of the phenol-bacterin vaccinated mice, and 19 per cen of the BPL-bacterin vaccinated mice survived.

Survival of vaccinated and unvaccinated guinea pigs after inoculation with M. bovis. The number of days was recorded at which vaccinated and unvaccinated guinea pigs

TABLE 3. The days on which vaccinated and unvaccinated mice died after intraperitoneal injection of 32.0 mg wet weight of Mycobacterium bovis.

Unvaccinated controls	Vaccinated[1] BCG	BPL	Phenol
4 @ 1[2]	4 @ 1		4 @ 1
1 @ 2		2 @ 2	2 @ 2
1 @ 5	1 @ 4	1 @ 4	
1 @ 7		2 @ 7	
		1 @ 9	1 @ 9
		2 @ 13	
	1 @ 14	4 @ 14	
1 @ 17			1 @ 17
	1 @ 18	1 @ 18	
1 @ 21	1 @ 21		
1 @ 24	2 @ 24		
10/11[3]	10/18	13/16	8/14

[1] Vaccinations were made with one mg BCG, 15 mg BPL-bacterin (BPL) and 15 mg phenol-bacterin (phenol)

[2] The first number indicates the number of mice which died and the second number indicates the day of death post-inoculation with virulent bacilli.

[3] The numerator indicates the number of animals which died and the denominator indicates the total number of animals inoculated.

TABLE 4. The mortality ratio and percentage survival of vaccinated and unvaccinated mice inoculated intraperitoneally with 32 mg wet weight of Mycobacterium bovis.

Days after inoculation		Vaccinated BCG	Vaccinated BPL	Vaccinated Phenol	Unvaccinated Control
6	death	5/18	3/16	6/14	6/11
	survival	72%	81%	58%	46%
7	death	5/18	5/16	6/14	7/11
	survival	72%	69%	58%	34%
10	death	5/18	6/16	7/14	7/14
	survival	72%	62%	50%	34%
24	death	10/18	13/16	8/14	10/11
	survival	45%	19%	42%	9%

[1] Intraperitoneal inoculations were made four weeks after vaccinations.
[2] Vaccinations were made with 1 mg BCG, 15 mg BPL-bacterin (BPL), and 15 mg phenol-bacterin (phenol).
[3] The mortality ratio is expressed as a fraction. The numerator indicates the number of animals which died and the denominator indicates the total number of animals inoculated.

died after intraperitoneal inoculation of two mg wet weight M. bovis (Table 5). On the 16th day post-inoculation, 40 per cent of the control and 80 to 100 per cent of the vaccinated guinea pigs were living (Table 6). The groups in which 100 per cent of the guinea pigs survived had been vaccinated with BCG, phenol-acetone-bacterin, 5 mg extract and 25 mg extract. The groups in which 90 per cent of the guinea pigs survived had been vaccinated with BPL-bacterin, BPL-acetone-bacterin, and 10 mg extract. The one group in which 80 per cent of the guinea pigs survived had been vaccinated with phenol-bacterin.

The only changes in the percentage of animals surviving on the 18th day post-inoculation were that there was 10 per cent less in the 5 mg extract, 10 mg extract and phenol-acetone-bacterin vaccinated guinea pigs and control guinea pigs.

All control guinea pigs had died 27 days after inoculation. The vaccinated guinea pigs survived as follows: 90 per cent with 25 mg extract, 89 per cent with BCG, 80 per cent with 5 mg extract, phenol-bacterin and BPL-bacterin, 70 per cent with 10 mg extract and BPL-acetone-bacterin and 60 per cent with phenol-acetone-bacterin.

Hypersensitivity of guinea pigs vaccinated with BPL-bacterin and phenol-bacterin. All guinea pigs vaccinated 6 weeks previously with BPL-bacterin and phenol bacterin, responded to tuberculin with an area of induration varying from 16 mm to 21 mm in diameter 48 hr after intradermal

TABLE 5. The days on which vaccinated and unvaccinated guinea pigs died after intraperitoneal injections of 2.0 mg wet weight of Mycobacterium bovis.

Unvaccinated controls	Vaccinated[1]							
	BCG	BPL	BPL-AC	Phenol	Phenol-AC	5 mg	10 mg	25 mg
	1 @ 2[2]							
			1 @ 10[3]				1 @ 12	
		1 @ 13						
2 @ 14				2 @ 14				
1 @ 15								
3 @ 16								
1 @ 18					1 @ 18	1 @ 18	1 @ 18	
					1 @ 19			
1 @ 19	1 @ 20							
			1 @ 21		1 @ 23	1 @ 23		
		1 @ 25	1 @ 25		1 @ 25		1 @ 25	1 @ 25
1 @ 26								
1 @ 27								
10/10[4]	1/9	2/10	3/10	2/10	4/10	2/10	3/10	1/10

1. Vaccinations were made with 0.5 mg BCG, 10 mg BPL-bacterin (BPL), 10 mg phenol-bacterin (phenol), 10 mg BPL-acetone-bacterin (BPL-Ac) and 10 mg phenol-acetone-bacterin (Phenol-Ac) and extract (5 mg, 10 mg, 25 mg).
2. Excluded due to early death.
3. The first number indicates the number of guinea pigs which died and the second number indicates the number of days post-inoculation with virulent bacilli.
4. The numerator indicates the number of animals which died and the denominator indicates the total number of animals inoculated.

TABLE 6. The mortality ratio and percentage survival of vaccinated and unvaccinated guinea pigs inoculated with 2 mg wet weight of Mycobacterium bovis.

Days after Inoculation[1]		Unvaccinated Controls	Vaccinated[2] BCG	BPL	BPL-Ac	Phen	Phen-Ac	5 mg	10 mg	25 mg
16	death[3]	6/10	0/9	1/10	1/10	2/10	0/10	0/10	1/10	0/10
	survival	40%	100%	90%	90%	80%	100%	100%	90%	100%
18	death	7/10	0/9	1/10	1/10	2/10	1/10	1/10	2/10	0/10
	survival	30%	100%	90%	90%	80%	90%	90%	80%	100%
27	death	10/10	1/9	2/10	3/10	2/10	4/10	2/10	2/10	1/10
	survival	0%	89%	80%	70%	80%	60%	80%	80%	90%

1. Intraperitoneal inoculations were made four weeks after vaccinations.
2. Vaccinations were made with 0.5 mg BCG, 10 mg BPL-bacterin (BPL), 10 mg BPL-acetone-bacterin (BPL-Ac), 10 mg phenol-bacterin (Phen), 10 mg phenol-acetone-bacterin (Phen-Ac), and extract (5 mg, 10 mg, 25 mg).
3. The mortality ratio is expressed as a fraction. The numerator represents the number of animals which died and the denominator indicates the total number of animals inoculated.

testing. The mean size of the reaction in the BPL-bacterin vaccinated animals was 19.0 mm. The mean size of the reaction in the phenol-bacterin vaccinated animals was 18.5 mm.

DISCUSSION

Preliminary studies of the inactivation of M. bovis with BPL. A number of points are to be made on the use of BPL for the inactivation of M. bovis. Under the proper conditions, BPL inactivated the organisms so that growth could not be initiated on an artificial medium. In addition, the guinea pigs which were inoculated with BPL-bacterin for the hypersensitivity study had not died four months after vaccination and no lesions were found at autopsy.

The final method selected for the preparation of the BPL-bacterin was the use of a 0.4 per cent of BPL with 3×10^7 cells per ml of distilled water incubated 2 hr at 37 C, during which time a pH of 7.6 ± 0.4 was maintained. A decrease in the concentration of BPL, an increase in the concentration of cells, a shorter incubation period, or failure to adjust the pH during the incubation resulted in the failure to inactivate all cells. Therefore, it is stressed that the use of BPL for inactivation of M. bovis requires that all factors must be standardized and maintained. In addition, it is known that medium constituents may hasten the degradation of BPL. If the cells were not washed and suspended in distilled water, this factor would also need to be considered.

It was established that under the conditions selected for the final method for the preparation of the BPL-bacterin, the concentration of the BPL was in sufficient excess to provide a satisfactory safety factor. Under those conditions, 0.1 per cent had inactivated all cells, and likewise, 0.4

per cent was able to inactivate a heavier suspension of cells. Therefore, a final concentration of 0.4 per cent BPL with the lesser concentration of cells provided an adequate safety factor.

The inactivation of bacteria with BPL possesses two advantages over the inactivation of viruses or sterilization of blood components with BPL. First, the bacteria may be washed prior to treatment and any components in the growth medium removed which may interefere with the action of BPL. Second, after the inactivation of the bacteria with BPL, the bacteria may be washed sufficiently to remove residual BPL and its degradation products which are toxic for animal tissues. A greater concentration of BPL may be used to provide a greater safety factor of inactivation without increasing the hazards due to toxicity or other adverse effects of BPL.

Evaluation of the tuberculoimmunity afforded mice and guinea pigs. The criterion for the demonstration of tuberculoimmunity was chosen as the difference in the percentage of vaccinated and unvaccinated mice which survived at a given time interval after the inoculation of a given amount of M. bovis. The choice of the amount of inoculum to be used was based on the mean number of days that unvaccinated animals survived after different amounts of M. bovis were injected intraperitoneally (Tables 1 and 2). Two factors were considered in the choice. First, an amount was desired which was small so that it would not overwhelm and mask any tuberculoimmunity conferred on the vaccinated animals.

Second, an amount was desired which was sufficiently large to insure death of the animals in a relatively short period of time, prior to the formation of gross lesions. The amounts of wet weight of M. bovis chosen were 4, 8, 16 and 32 mg for mice and 2.0 g for guinea pigs. Owing to laboratory error, the results of the mice inoculated with 4, 8, and 16 mg wet weight of M. bovis could not be included.

The time interval at which the comparison of the percentage of vaccinated and unvaccinated mice survived was not selected prior to the inoculations. The percentages are noted at different time intervals and confirm the observations of other workers that the reliability of the evaluation of tuberculoimmunity is limited by the criterion chosen. For example, the interpretations may be biased by the number of days after inoculation which is chosen as a point of comparison of the percentage of animals surviving. If the day is chosen at which approximately one half of the control mice have died, the sixth day after inoculation, the tuberculoimmunity afforded the mice which received the BPL-bacterin is superior to that afforded by BCG or the phenol-bacterin. The percentage of mice surviving was 81, 72 and 58. respectively.

On the 10th day after inoculation, 34 per cent of the control mice lived. More of the mice which had received the BPL-bacterin survived than those vaccinated with the phenol-bacterin but less than those vaccinated with BCG. The percentage of mice surviving was 62, 50 and 72, respectively.

On the 24th day after inoculation, only 10 per cent more of the BPL-bacterin vaccinated mice survived than the control mice, 19 and 9 per cent, respectively. Mice vaccinated with BCG or the phenol-bacterin survived, 45 and 42 per cent, respectively. Both were markedly superior to the BPL-bacterin at this time interval.

Bloch and Segal (1955) obtained such equivocal results. They found that control mice survived longer than mice vaccinated with BCG. The authors believed that this was due to the differences in the distribution of lesions containing virulent bacilli. Animals sensitized by the vaccination with BCG developed more extensive tubercles than the controls which had not been previously sensitized.

Criteria other than survival have been used to measure tuberculoimmunity, such as differences in lung densities, numbers of lesions, or the number of bacilli culturable from a given organ (Feldman, 1943; Crowle, 1958a). These criteria have been criticized by Youmans (1957) in that individual animal susceptibility varies markedly and greatly influences the results. Survival of a group of animals is the most reliable but as noted previously, the interpretation of the results may be heavily biased by the time interval selected. The longer the interval, the more complicated the evaluation becomes due to the complexity of the interplay of the development of infection and hypersensitivity after challenge with the hypersensitivity previously induced by vaccination. Shorter time intervals are believed to be more reliable.

The amount of organisms used to challenge experimental tuberculoimmunity undoubtedly far exceeds that which occurs naturally and by virtue of the smaller number of organisms, would bring into play less untoward reactions during natural infections.

Weiss and Dubos (1954) stressed that all mycobacterins are toxic to some degree and animals which survive the toxic effects before their immunity is challenged, are comparatively resistant.

After the vaccination of the mice, some toxic effects of the extract were evident. Approximately 50 per cent of the mice died before their immunity could be challenged and for this reason, the results of mice vaccinated with the extract could not be included. In preference to repeating the experiment in mice, guinea pigs were chosen on the basis that they are more susceptible to infection with M. bovis.

The measurements of the relative tuberculoimmunity were not as markedly altered by the selection of different time intervals for the guinea pigs (Table 5) as those for the mice (Table 3). It was apparent that all groups of vaccinated guinea pigs had been protected if the day at which approximately one half of the control guinea pigs had died, the 16th day post-inoculation were chosen, or the 18th day, which was the mean day, or the 27th day when all guinea pigs had died. The vaccinated pigs surviving at the 16th and 18th day varied from 80 to 100 per cent, and at the 27th day, 60 to 90 per cent.

In some instances, the immunity afforded by the vaccine, a given bacterin or concentration of extract appeared to be superior to others. Consistently, BCG and 25 mg extract conferred the greatest protection upon guinea pigs. Initially, 5 mg extract and phenol-acetone-bacterin were equally effective but by the 27th day, 5 mg had protected 10 per cent less and phenol-acetone-bacterin was the poorest of the lot.

The tuberculoimmunity afforded by the BPL-bacterin was not as great as that afforded by BCG and 25 mg extract but was consistently superior or equal to that of phenol-bacterin and 10 mg extract.

Of interest are the acetone washed BPL-bacterin and phenol-bacterin. Acetone soluble fractions, as an extract or part of the cell, reportedly are toxic for experimental animals and are believed to increase the animal's susceptibility to M. bovis (Weiss and Dubos, 1954; Crowle, 1958a). On this premise, it could be anticipated that the acetone washed bacterins should be more effective in inducing tuberculoimmunity than the unwashed bacterins. This proved to be true of phenol-acetone-bacterin at the 16th and 18th day, but fewer guinea pigs vaccinated with the phenol-acetone-bacterin survived at the 27th day than those vaccinated with phenol-bacterin. Likewise, fewer guinea pigs vaccinated with BPL-acetone-bacterin survived at the 27th day than those vaccinated with BPL-bacterin although they were equally effective at the 16th and 18th day.

Kanai and Youmans (1960) reported that 10 mg of an extract of avirulent M. tuberculosis afforded mice better protection than 5 mg or 25 mg. It was demonstrated in the present investigation that 5 mg and 25 mg of the extract from virulent M. bovis were more immunogenic for guinea pigs than 10 mg. The unpredictability of the optimum dose is a distinct disadvantage in the use of the extract.

The extract prepared by Kanai and Youmans (1960), which was highly immunizing for mice, was prepared from M. tuberculosis. It has been demonstrated by the present experiment that an extract from M. bovis can confer a tuberculoimmunity on guinea pigs equal to, or better than, the intact bacilli inactivated with phenol or BPL. It is suggested that similar extracts be made from saprophytic mycobacteria to determine if they possess tuberculoimmunogenic properties. Saprophytic mycobacteria grow more rapidly and would be less hazardous to handle.

The ideal immunization procedure would be one which would induce a significant tuberculoimmunity without also inducing tuberculin sensitivity. The value of the tuberculin test as a diagnostic tool would be retained. The extract reportedly does not induce sensitivity, and as such, is highly desirable. Unfortunately, massive amounts of cells, by a tedious and hazardous procedure, yields minute amounts of extracts. From approximately 200 ml of packed cells, 600 mg extract was obtained.

The BPL-bacterin retained the ability to confer a

degree of tuberculoimmunity and also hypersensitivity. Extractions to remove the wax fractions, which with the proteins reportedly induce the sensitivity, may yield a product capable of immunization without sensitization.

This study was undertaken originally to answer two questions, the second contingent upon the results of the first. The questions were 1) will BPL inactivate M. bovis, and if so, 2) will BPL-inactivated M. bovis cells be tuberculoimmunogenic? An affirmative answer has been obtained to both questions. Under the proper conditions, M. bovis is inactivated by BPL. The organisms so inactivated are tuberculoimmunogenic. However, the tuberculoimmunity conferred is relative and not absolute. The difficulty of interpreting data has been noted. It has been measured not only by the differences in the susceptibility to M. bovis of mice and guinea pigs unvaccinated and vaccinated with BPL-bacterin, but also animals vaccinated with BCG, phenol-inactivated cells and extracts of cells. The former, BCG, has been used world-wide, reportedly successfully. It is known that the organism resides for long periods of time in the animal tissues after vaccination, and undoubtedly multiplies. Thus, equal numbers of BCG and cells of a bacterin are not, in reality, equal numbers after inoculations. A comparison of the two is unfavorably biased toward BCG. However, no hazard from infection exists in the use of a bacterin. Therefore, both points must be weighed.

A second point is to be made in comparing a bacterin to

a vaccine. The role of circulating antibodies in tuberculoimmunity is not known. Their presence or absence can not be correlated conclusively with a given stage of the disease or serve as a basis for prognosis. It has been suggested that tuberculoimmunity is dependent upon some intracellular activity of the host cells which retard the growth of the bacillus, and that antigen-antibody reactions are incidental. If this be true, it is possible that by multiplication in the host, BCG elicits a greater response which can never be equalled by a bacterin. If the stimulating substance is retained in the bacterins, the bulk of the bacterin may contribute very little to induction of tuberculoimmunity. However, the little that is known of the mechanism of infection, immunity or hypersensitivity of tuberculosis is testimony of the complexities involved and an indication that no one single factor controls one or all conditions.

Mycobacterium bovis is inactivated by BPL. Such organisms may serve as a source of non-viable cells from which extracts can be prepared in large volumes without hazards. The tuberculoimmunity and sensitivity detected in guinea pigs after vaccinations with BPL-bacterin establish that the antigenicity is retained in the bacterin. Such antigens may possess more potential species differentiation, if it exists, than those commonly used in serologic, identification, and sensitivity studies.

SUMMARY

Beta-propiolactone (BPL) inactivated Mycobacterium bovis. A final concentration of 0.1 per cent of BPL was required to inactivate 3.4×10^7 cells per ml of distilled water when incubated at 37 C for 2 hr at pH 7.6 ± 0.4. All cells were not inactivated if the concentration of BPL was decreased, the concentration of the cells increased, the incubation period was shortened to 1 hr or if pH 7.6 ± 0.4 was not maintained.

A BPL-bacterin prepared from M. bovis was found to be a safe, effective tuberculoimmunogenic agent for mice and guinea pigs. The BPL-bacterin was made by treating 3×10^7 cells with a final concentration of 0.4 per cent BPL. The excess BPL provided a satisfactory safety factor. The inactivated cells had to be washed four times to remove any residual BPL or degradation products toxic for the experimental animals.

The tuberculoimmunity elicited in mice by BPL-bacterin compared favorably to that elicited by BCG vaccine and was superior to that elicited by phenol-inactivated cells. When approximately two-thirds of unvaccinated mice had died after the inoculation of virulent organisms, 72 per cent of BCG vaccinated mice, 69 per cent of BPL-bacterin vaccinated mice and 58 per cent of phenol-bacterin mice survived.

Tuberculoimmunity was elicited in guinea pigs by the vaccine, bacterins and extract. After challenge with virulent M. bovis, when two thirds of control animals had died, all guinea pigs

vaccinated with BCG and 25 mg extract, 90 per cent of BPL-bacterin and 5 mg extract vaccinated guinea pigs, and 80 per cent of phenol-bacterin and 10 mg extract vaccinated guinea pigs survived. Treatment of the BPL-bacterin and phenol-bacterin with acetone before administration did not increase the protection afforded the guinea pigs.

An extract was obtained from M. bovis by a method similar to that by which Youmans (1960) prepared an extract from M. tuberculosis. The extract of M. bovis conferred tuberculoimmunity to guinea pigs which was assumed to be similar to that conferred to mice by the extract of M. tuberculosis. Extracts from saprophytic mycobacteria which grow more readily and are less hazardous would be worthy of investigation.

Tuberculin sensitivity was induced in guinea pigs by BPL-bacterin. Extractions of waxes from the bacterin may yield a product which would induce tuberculoimmunity without delayed hypersensitivity.

Extractions may be made of virulent mycobacteria inactivated with BPL for identification, serologic and sensitivity studies.

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