# THE SEROLOGICAL RESPONSE IN RABBITS TO LIVE, IRRADIATED AND HEAT-KILLED MYCOBACTERIAL VACCINES

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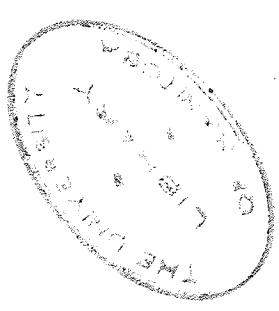

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# THE SEROLOGICAL RESPONSE IN RABBITS TO LIVE, IRRADIATED, HEAT-KILLED AND COMPOSITE MYCOBACTERIAL VACCINES

By Ivan Kochan

## ABSTRACT

Antibody formation in four groups of five rabbits after vaccination with live, irradiated, heat-killed or composite BCG vaccine was measured, over a period of 8-14 weeks, using the hemagglutination test of Middlebrook and Dubos. Hemagglutinins varied in titer and in duration with each of the vaccines employed. The range in titer was 0-1:256 in series I with live vaccine, 0-1:256 in series II with irradiated vaccine, 0-1:64 in series III with heat-killed vaccine and 0-1:32 in series IV with composite vaccine. A mean titer of 1:16 or higher was maintained in series I for eight weeks, in series II for 11 weeks, in series III for three weeks and in series IV for one week. The serological response in four rabbits to a series of intravenous injections of heat-killed tubercle bacilli was much higher in titer than to a single percutaneous vaccination. The results indicate that the hemagglutination test may be useful for estimating the degree of serological response, and consequently for evaluating various mycobacterial vaccines.

In an attempt to develop a standard hemagglutination

procedure for this work, it was found that the Hull O.T. (human) was a more effective sensitizing agent than the other Old Tuberculins tested and that phosphate buffered saline as a diluent was more satisfactory than buffered isotonic saline. Sera inactivated before storage and sera inactivated after storage produced essentially the same titer.

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FIGURE

1. Mean serological response as measured by hemagglutinin level in rabbits vaccinated with: I. A live mycobacterial vaccine; II. An irradiated mycobacterial vaccine; III. A heat-killed mycobacterial vaccine; IV. A composite mycobacterial vaccine. 38

# INTRODUCTION

In the study of tuberculosis in the past and particularly in recent years, while prophylaxis and therapy have advanced extensively, immunity has not undergone a parallel development. A specific and sensitive serological test is required for determining the degree of induced immunity, for diagnosis and for assessing the extent of tuberculous lesions. For these purposes agglutination, precipitation and complement fixation tests have been applied and were discarded in the course of time because the methods proved to be unreliable. In 1948 Middlebrook and Dubos (39) revived interest in the serological aspects of tuberculosis by developing a method based on specific serum agglutination of sheep's red blood cells previously treated with extracts of bacilli. The procedure, generally referred to as the hemagglutination test, was recommended for determining serological response in the disease. For the most part the test has been investigated with regard to its clinical application and results indicate that it is diagnostically not significant. However, more specific and consistent antibody levels were determined by the hemagglutination test than by any other serological reaction. For this reason the test offers another means for the assessment of response to vaccination and for the study

of the basic aspects of immunology.

Various prophylactic vaccination methods employed from the time of Koch's demonstration of tubercle bacilli (34) have been considered not satisfactory. In 1922 Calmette and Guerin (11) recommended the use of an attenuated bovine strain for vaccination and most authorities agree that the strain, BCG, has some protective value as determined by survival tests and epidemiological studies. The ever present possibility of the resumption of virulence within the body by attenuated organisms has prompted some workers (55, 43, 42, 49) to advocate vaccines prepared from killed tubercle bacilli.

This study was undertaken to determine the immunological response, as measured by the hemagglutination test, to vaccination in rabbits with live, irradiated and heat-killed tubercle bacilli.

# HISTORICAL

The Great White Plague of two thousand years ago was put on a sound scientific basis by R. Koch when, in March 1882, he reported the isolation of the tubercle bacillus and presented experimental evidence proving its infectious nature. Since then many workers have attempted to prevent the disease by immunization with vaccines prepared with live, killed or attenuated bacilli and by applying different techniques. Serological methods were employed for the evaluation of immunization as well as for the diagnosis of tuberculosis, but were discarded in the course of time because of their lack of specificity. The protection test, which measured the survival time of a vaccinated animal to a lethal dose of tubercle bacilli, was used for the estimation of resistance against the disease. The lack of a method to measure the serological response over a period of time has hindered the development and understanding of the basic immunological problems in tuberculosis.

## Mycobacterial Vaccines.

The first attempts to produce resistance were confined almost exclusively to the use of weakened human and bovine types of tubercle bacilli. Grancher and Martin (26) used

bovine bacilli attenuated by prolonged growth on artificial media for the vaccination of rabbits. Several subcutaneous injections were made in series, each injection containing younger and thus more virulent bacilli. After an interval, the vaccinated rabbits which were injected with virulent tubercle bacilli survived longer than the controls. Pearson and Gilliland (44) showed that different strains varied in immunological properties, as for example avian bacilli did not confer significant immunity upon cattle. Also, they pointed out that human tubercle bacilli incubated in a collodion capsule in the abdominal cavity of a bull for seven months protected cattle against the human virulent strain. These workers therefore advocated vaccination of cattle with living cultures of low virulence to control the disease in animals. A significant investigation with attenuated organisms was carried out by Behring (5) who maintained a human type culture for six and one-half years, after which time the virulence for guinea pigs was greatly reduced. Cultures prepared from this weakened strain were dried and administered intravenously to cattle. The animals developed definite resistance to pathogenic organisms which were subsequently injected. Von Behring later was less certain of the value of his vaccine, which he had called "bovovaccine", because the protection obtained was of short duration and because several animals developed tuberculosis as a result of vaccination. Other workers modified von Behring's tech-

nique, generally reducing virulence by physical or chemical methods. Levy et al (35) tried to attenuate cultures with urea and reported that bacilli treated with 25% solution of urea for two days were non-pathogenic and were satisfactory immunizing agents. Klimmer (33) obtained protection in cattle for one year by administering a composite vaccine of tubercle bacilli attenuated by heat and tubercle bacilli attenuated by successive passage through salamanders. Many workers attenuated bacilli by other methods, but none of the preparations induced more than temporary immunity.

Calmette and Guerin (12), after unsatisfactory results with dead tubercle bacilli, referred back to von Behring's work on the production of immunity in animals by living organisms of attenuated virulence. They proposed the hypothesis that a selected attenuated strain showing permanent non-pathogenic characters would immunize animals and humans. A highly virulent bovine type of tubercle bacillus, isolated from the udder of a tuberculous cow, was cultured by Calmette and Guerin on a glycerin-bile-potato medium over a period of 13 years. At the end of that period its virulence for all species of animals was low and vaccination with Bacillus Calmette-Guerin, generally referred to as BCG, induced considerable immunity. The protective effect in animals led Calmette and Guerin to use the vaccine on human subjects and to recommend the vaccine for newborn infants (11).

BCG vaccination has been carried out on an extensive

scale with no striking results and the question of its prophylactic value remains unsettled. On the one hand certain workers maintain that BCG vaccination provides an effective control of tuberculosis. On the other hand other workers believe that the attenuated strain offers no protection and that it may even be the cause of clinical tuberculosis. After 43 years in animals and 33 years in humans most authorities consider BCG vaccination still to be in the investigational stage. It is generally accepted that some protection is conferred but there is no agreement that it should be adopted as a general public health measure.

On July 12, 1950, the United States Public Health Service licensed the Research Foundation and the University of Illinois for the "manufacture, exportation, importation and sale" of BCG (2). The vaccine produced by this laboratory was found to be safe by trial with animals; it was free from contaminating substances and produced a satisfactory and immediate reaction in animals and humans when used within the prescribed time limit. This strain was recommended for vaccination of humans who came in contact with tubercle bacilli in their occupation or at home.

The first attempts to develop immunity to tuberculosis by using virulent organisms were made by Webb and Williams (64) by vaccination with minute doses of the organisms, beginning with one cell and progressively increasing the number of bacilli. At nine months after the last injection no tubercu-

lous lesions were found in the experimental animals. Calmette and Bruyant (12), using this method, showed that animals did develop discrete tuberculous lesions after 12-18 months. These results prompted them to emphasize the danger of using virulent tubercle bacilli, even in minute dosages.

While extensive studies were carried out to produce immunity by vaccination with living, attenuated or virulent tubercle bacilli, some workers believed that the organisms should be killed before introduction into the animal or human body. Various physical and chemical lethal agents were tested to determine which would bring about the least change in the protective properties of the bacterial cell.

Heat was used to kill tubercle bacilli by Dembinski (17). Several increasing doses of the non-viable bacilli were injected into rabbits which developed some resistance to virulent organisms. Calmette, Guerin and Breton (12) fed guinea pigs heat-killed bacilli and concluded that the treatment produced partial immunity. Raw (51) prepared a vaccine from attenuated organisms that were later heat-killed for the vaccination of humans and reported excellent results.

After the early attempts to produce a heat-killed vaccine, attention was directed primarily to the viable, attenuated strain of Calmette and Guerin. Petroff et al (46) grew the BCG on a gentian violet-egg medium and observed a virulent variant which produced progressive tuberculosis in guinea pigs. As this finding was confirmed by other

workers (60, 61) the use of killed tubercle bacilli was revived and to some extent persists to the present day. Petroff and Steenken (47) compared the resistance established by a living BCG vaccine with a killed bacillus vaccine and maintained that the protection induced by living BCG was neither more efficient nor safer than that caused by heat-killed organisms. For that reason Petroff et al (45) advocated vaccines prepared from heat-killed bacilli for the immunization of children. Opie and Freund (42) showed that heat-killed bacilli induced increased resistance to infection, which was only slightly less than that produced by living BCG. On the other hand Copper et al (15) reported that infection of guinea pigs with living, avirulent bacilli retarded subsequent infection with virulent organisms, but that heat-killed avirulent or virulent bacilli exerted no such effect.

Since heat has severe effects on the protoplasm of microorganisms, a less destructive agent such as ultraviolet rays was recommended by Burger (8) for the killing of bacilli for the preparation of superior vaccines. Olson et al (41) also showed that a virulent strain of mycobacteria killed by ultraviolet light was a more effective vaccine than heat-killed bacilli; and was superior to a BCG strain which had been rendered nonviable by ultraviolet light. Sarber et al (55) showed that a vaccine from virulent tubercle bacilli, killed by ultraviolet irradiation, possessed antigenic values equal to BCG on the basis of the guinea pig protection test.

Several workers produced vaccines by treating tubercle bacilli with chemicals such as Javelle water, iodine water, sodium fluoride, oleic acid etc. Any resistance that developed was fleeting or did not appear at all. For example Deycke and Much (18) treated mycobacteria with ovolecithin and obtained partial protection in animals. Calmette and Breton (12) used chlorinated bacilli which failed to protect animals against an infective dose. Branch and Enders (7) vaccinated guinea pigs with formol-killed and with heat-killed organisms and after one year tested them for protection. The heat-killed vaccine was more effective than the formol-killed but the difference was not pronounced.

Potter (49) suggested "bacterial asphyxia" for the preparation of a killed tubercle bacillus vaccine in place of heat or chemicals. He incubated the tubercle bacilli in a buffered solution for one month at 38-40°C in partial vacuum, saturated with water vapor but deprived of oxygen. Potter reported very favorable results and it is surprising that this method has not been further investigated.

Serological Response to Tubercle Bacilli.

In tuberculosis, as in other infectious diseases, the production of antibodies is a response to contact with antigens and can be measured by various serological tests.

The detection of antibodies in tuberculosis was first carried out by Arloing and Courmout (3) with the agglutination

of live or killed tubercle bacilli in twofold serial dilutions of sera. A positive reaction showed microscopically visible flakes and the highest dilution of serum showing agglutination was designated as the titer. The test was considered to be diagnostically significant. However, it was later shown that factors, such as origin of the bacilli, concentration, and the medium on which cultivated influenced the titer. Many variable results with agglutination have since been reported. Simintzis and Sohier (57) reported inconsistent results, with non-tuberculous sera frequently giving a high agglutination titer and tuberculous sera giving normal titers. For this reason, the specificity and the value of the test for practical purposes has been questioned.

The complement fixation reaction has been investigated and until recently was widely used for the detection of antibodies in tuberculous sera. Early workers, basing their methods on the complement fixation of the Wassermann reaction for syphilis, were successful in measuring antibodies in tuberculosis, but concluded that a more selective antigen was necessary to increase the specificity and sensitivity of the test. In search for such an antigen Calmette and Massol (14) tested two preparations which they designated B-I and B-II. The B-I was prepared by maceration in distilled water at 65°C and the B-II by maceration in 10% peptone "Witte" solution. With either antigen 33-50% positive results were obtained with the sera of tuberculous patients.

Wassermann (63), to prepare antigen which would not fix the lipoidal antibodies present in syphilitic sera, employed defatted bacilli which were washed in ether and dried to a powder. A suspension of the "powder" in saline yielded albuminous substances, which were used for the complement fixation reaction with tuberculous sera. However, it did not prove to be sufficiently sensitive. Wassermann therefore added lecithin to the specific albuminous antigen, which increased the sensitivity and did not lower specificity. This antigen was recommended and widely used in the test. Numerous workers have prepared other antigens. Maltaner and Wadsworth (62) examined the complement fixation test and found that the reaction was positive in 85-95% of the sera of patients with pulmonary tuberculosis and that the titer varied according to the stage and type of disease; in chronic cases the reaction was generally positive, but in acute cases such as miliary or tubercular meningitis negative. In recent years because of inconsistent results the clinical application of the complement fixation has been largely discounted.

The precipitation test was first studied by Massol (13) with bovine immune sera mixed with various types of tuberculin. In the same year it was shown by Bezançon and Serbonnes (6) that sera of pneumonia and typhoid fever patients when mixed with tuberculin also gave marked precipitation. Porter (48) demonstrated that the test was very often positive with sera of healthy people. It appeared, therefore, that the preci-

losis but was usually negative in patients with far advanced disease. More recent reports such as that by Fleming et al (23) showed no close correlation with active tuberculosis but the titers with sera of tuberculous patients tended to be higher than normal. Kirby et al (32) contended that the test was of little practical value in the diagnosis of tuberculosis, because 10% of non-tuberculous patients gave positive reactions to the test. A similar conclusion was reported by Hollander et al (31) who found that sera of 17% of the patients with clinically active pulmonary tuberculosis showed negative hemagglutination reactions. In the study of the effect of BCG vaccination Smith and Scott demonstrated that 77% of vaccinated humans had positive reactions. Haley and associates (27), by using the hemagglutination test, found that a group of 166 BCG vaccinated persons developed an antibody response that was quantitatively of a very low order and was transient.

## PRELIMINARY STUDIES

The hemagglutination test outlined by Middlebrook and Dubos (39) served as a basic method to which workers added modifications in order to increase its sensitivity and effectiveness. For this reason a few variations are present in each phase of the method. Because the test was the means by which serological response was to be tested in this work, preliminary studies were carried out on some of these modifications before proceeding with the main investigation. In each experiment a standard method later outlined under Material and Methods was followed, unless otherwise indicated.

(1) Type of Old Tuberculin.

An extract of tubercle bacilli was employed by Middlebrook and Dubos (39) to sensitize erythrocytes to specific immune sera. For the same purpose in a modified hemagglutination method Scott and Smith (56) recommended Old Tuberculin, a 1:15 dilution of four times standard strength (Lederle). These workers estimated that a dilution 1:8 or higher rendered the 4 x O.T. nonhemolytic to red cells and that complete sensitization of erythrocytes took place up to 1:20. Rothbard and associates (54) used a 1:12 dilution of the Lederle tuberculin and this concentration has been