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

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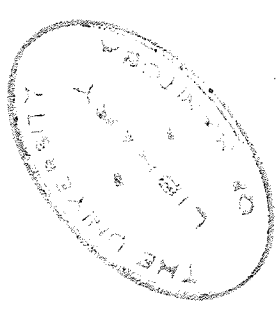

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ABSTRACT

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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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-

pitation test could not be used for the diagnosis of tuberculosis or for measuring the antibody production in immunized animals with the techniques available.

It is known from the studies of Burnet and Anderson (9, 10) that red blood corpuscles can absorb various substances and are thereby rendered specifically agglutinable by the serum directed against the substances absorbed (52). Middlebrook and Dubos (39) applied this principle to develop a method which demonstrated the specific serum agglutination of erythrocytes sensitized with extracts of mycobacteria. A substance or substances present in the bacillary extract was absorbed by erythrocytes, sensitizing them so that they agglutinated in the presence of antibodies to tubercle bacilli. The results obtained indicated that the reaction was more specific than other serologic procedures and that a positive test might suggest a tuberculous infection. Preliminary reports on the method were encouraging. Rothbard and associates (54) made a study of the hemagglutination reaction on 216 non-tuberculous patients, 33 cured tuberculous persons and 168 patients with active tuberculosis. A negative reaction was observed in 203 cases of the first group and 31 of the second group. One hundred and fifty four of the third group showed a positive reaction in a titer of 1:8 or higher. This was highly suggestive of active disease. In the same year Smith and Scott (58) reported that the hemagglutination test was generally positive in patients with active tubercu-

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

employed by many workers.

In this study the sensitizing property of four brands of Old Tuberculin were compared by the hemagglutination test on sera obtained from rabbits vaccinated with attenuated tubercle bacilli in an earlier study.

(a). Human type tuberculin, 25% O.T. (Human strain PN, DT & C). Hull. Diluted in 1:83 in phosphate buffered saline.

(b). Concentrated tuberculin 4 x International standard. Lederle. Diluted 1:12.

(c). Bovine type tuberculin, 50% O.T. (Bovine strain Sp. 28). Hull. Diluted 1:40.

(d). "Wellcome" Old Tuberculin (T). Diluted 1:60.

Twenty eight sera were tested with erythrocytes sensitized by (a); the same 28 by (b); 12 of the 28 by (c) and the remaining 16 of the 28 by (d).

The titers in (a) ranged from 0-2048. Eleven of the 28 ranged from 0-1:8; 13 from 1:16-1:128 and four from 1:256-1:2048 inclusively. Using (b) 18 of the 28 sera showed the same titer as in (a); six were higher and four lower. Only one serum showed greater than a twofold difference in titer. Of the 12 sera using (c) six showed the same titer, three were higher and three lower as compared to (a). Of 16 sera tested with (d) seven showed the same titers as in (a); two were higher and seven lower. Three of the sera with lower titers showed a difference greater than a twofold dilution.

TABLE I

ANTIBODY TITERS OF THREE TYPES OF O.T.,
EACH COMPARED WITH HULL O.T. (HUM.)

Titer Range	(a) Hull O.T. (Human)	(b) Lederle O.T.			(a) Hull O.T. (Human)	(c) Hull O.T. (Bovine)			(a) Hull O.T. (Human)	(d) Wellcome O.T.		
		Same	Higher	Lower		Same	Higher	Lower		Same	Higher	Lower
0-1:8	11[x]	8	2	1	5	3	1	1	8	4	2	1,1a[xx]
1:16-1:32	13	8	2,1a	2	4	2	0	2	7	3	0	3,1a
1:256-1:2048	4	2	1	1	3	1	2	0	1	0	0	1a
Total	28	18	5,1a	4	12	6	3	3	16	7	2	4,3a

[x] Figures represent numbers of sera in each titer range.
[xx] "a" indicates a difference of more than one titer.

A titer difference of twofold dilution may be considered within the experimental error. On this basis no difference was observed with (b) and (c). Only with (d) were lower titers observed. No difference was observed with any of the tuberculins in the proportion of sera in the various titer groups. The data are presented in Table I.

The clumping of red cells sensitized with Hull O.T. (human) was much easier to read than agglutination with the other tuberculins, the clumps were larger and not easily dispersible. For these reasons the Hull O.T. (human) was adopted for the sensitization of erythrocytes and was used throughout the study.

At about the same time and with other immune sera the sensitization property of Purified Protein Derivative (P.P.D.) was investigated with solutions containing 0.2 mgm., 0.02 mgm. and 0.01 mgm. per ml. Six sera were used to test each concentration. Hemolysis was obtained with the cells sensitized with 0.2 mgm. and with 0.02 mgm. of P.P.D. per ml. Hemolysis did not occur with 0.01 mgm. per ml. but the cells showed no evidence of clumping.

(2) Preservation of Sera.

In general practice sera are stored at freezing temperatures to prevent deterioration and to preserve their specific immunological properties. Throughout this study sera were kept at -20°C and thawed at room temperature on

the day of testing.

The effect of storage was determined on two groups of sera stored for different periods of time. In the first group the titers of 19 sera determined after three months were compared to titers obtained after 2-4 days. In the second group the titers of 34 sera determined after 18 months were compared to titers obtained after 2-6 days storage. The standard hemagglutination procedure was followed in both cases.

The titers of both groups are presented in Table II. The range of titers in the 19 sera of the first group stored for 2-4 days showed three samples from 0-1:8, eight samples from 1:16-1:32 and eight samples from 1:64-1:128. After storage for three months, 15 of the 19 sera showed the same titers with one higher and three lower than originally. The differences were twofold only and therefore were considered to be not significant.

The titers of 34 samples of the second group showed 14 which ranged in titer levels from 0-1:8, 12 from 1:16-1:32 and eight from 1:64-1:128. After 18 months' storage, three samples maintained the same titers, one serum was higher and 30 were lower than originally. The difference in the latter category in 11 samples was twofold lower and in 19 threefold to sixfold lower.

The results on the two groups of sera show that storage for three months did not alter the antibody levels

TABLE II

THE EFFECT OF STORAGE OF SERA ON HEMAGGLUTINATION TITER

(1) Stored 3 months

Titer Range	Stored 2-4 Days	Same	Higher	Lower
0-1:8	3[x]	2	0	1
1:16-1:32	8	7	1	0
1:64-1:128	8	6	0	2
Total	19	15	1	3

(2) Stored 18 months

Titer Range	Stored 2-6 days	Same	Higher	Lower
0-1:8	14	2	0	6,6a[xx]
1:16-1:32	12	1	0	4,7a
1:64-1:128	8	0	1	1,6a
Total	34	3	1	11,19a

[x] figures represent numbers of samples in each titer range.

[xx] "a" indicates difference of more than one twofold dilution.

but storage for 18 months showed significantly lower titers.

It is interesting to note that the hyperimmune serum, prepared by the method of Rothbard and associates (54), showed the same titer (1:2048), even after 22 months' storage.

(3) The inactivation of complement in sera before and after the storage.

In the method of Middlebrook and Dubos (39) the immune sera were stored without the inactivation of complement, which was carried out before testing. Inactivation before storage has been advocated by Elek (22), who observed that fresh sera stored in the unheated state lose a considerable amount of antibodies.

TABLE III

EFFECT OF TIME OF COMPLEMENT INACTIVATION ON ANTIBODY LEVELS OF IMMUNE SERA

Titer Range	Before Storage	Stored 14 days		
		Same	Higher	Lower
0-1:8	8[x]	5	2	1
1:16-1:32	6	3	2	1
1:64-1:2048	3	2	0	1a[xx]
Total	17	10	4	2,1a

[x] figures represent numbers of samples in each titer range.

[xx] "a" indicates difference of more than one twofold dilution.

In this study the effect of complement inactivation, before storage for 14 days, was investigated. Seventeen samples of freshly obtained sera were divided into two portions; the first was inactivated in a water bath at 56°C for 30 min. and then both were stored at -20°C. After 14 days the second portion was inactivated and the hemagglutination test was then performed on both portions.

The results presented in Table III show that of 17 samples inactivated before storage eight titers ranged from 0-1:8, six from 1:16-1:32 and three from 1:64-1:2048. The samples inactivated after storage, when compared to those inactivated before storage, showed 10 with the same titer, four twofold higher and three twofold lower. Only one sample showed a difference greater than one twofold dilution.

The results, therefore, indicate no perceptible effect of complement inactivation before storage on antibody levels of immune sera.

(4) Buffered isotonic saline and phosphate buffered saline.

Buffered isotonic saline has been employed as a diluent for the hemagglutination test by many workers (4, 56), with some attempts to improve its buffering and electrolytical properties (30). Phosphate buffered saline was used by Mollow and Kott (40) in the comparative study of the hemagglutination test and its hemolytic modification.

Phosphate buffered saline was compared to buffered

isotonic saline on the basis of the hemagglutination test performed on 12 sera. On each serum two tests were carried out; in the first the phosphate buffered saline and in the second the buffered isotonic saline was used for all diluting purposes. Both tests were run concomitantly.

TABLE IV

EFFECT OF DILUENT ON TITERS OBTAINED IN THE HEMAGGLUTINATION TEST

Titer Range	Phosphate Buffered Saline	Buffered Isotonic Saline		
		Same	Higher	Lower
0-1:8	2[x]	1	0	1
1:16-1:32	6	1	0	1,4a[xx]
1:64-1:256	4	1	0	2,1a
Total	12	3	0	4,5a

[x] figures present numbers of samples at each titer range.
[xx] "a" indicates a difference of more than one twofold dilution.

The titers with phosphate buffered saline showed two sera ranging in titers from 0-1:8, six from 1:16-1:32 and four from 1:64-1:256. With buffered isotonic saline, three sera showed the same titer, no sample was higher and nine

were lower. The titer was twofold lower in four, and threefold lower in five sera.

The results indicate that phosphate buffered saline gave increased sensitivity to the test and it was employed throughout the study.

MATERIAL AND METHODS

Vaccines.

The vaccines were prepared from a strain of BCG, No. 867-S_2 of Mycobacterium tuberculosis, received from the Department of National Health and Welfare, Ottawa. An actively growing culture was grown in tween-albumin medium (21) for ten days, at which time about 15 mgm. of bacilli, dry weight, per ml. were present. The microorganisms were washed three times in 25% Sauton's medium (21) and were re-suspended to give 30 mgm. dry weight per ml. The number of living bacilli was estimated by a viability test which consisted of inoculating aliquots of the 30 mgm. suspension on five slants of Petragnani's medium (38). After incubation at 37.5°C for eight weeks the colony count showed 10^8 to 10^9 bacilli per ml.

The 30 mgm. suspension was employed to prepare live, irradiated, heat-killed and composite vaccines.

I. Live vaccine: The suspension was used in an unaltered state.

II. Irradiated vaccine: The bacilli were irradiated by ultra-violet light at 10 cm. for three min. The source of radiation was a mercury vapor resonance lamp of 3130A° units[x].

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[x] Hanovia Chemical Co. Newark, New Jersey.

The suspension was shaken during the process to ensure that all cells received the same exposure. The number of survivors was determined by the viability test in 10^{-1}, 10^{-2} and 10^{-3} dilutions.

III. Heat-killed vaccine: The suspension of bacilli was heated in a water-bath at 60°C for 30 min. The viability test was carried out using the same dilutions as in the irradiated vaccine.

IV. Composite vaccine: The vaccine was composed of both heat-killed and viable bacilli in a concentration of 30 mgm. of cells per ml. The killed bacilli constituted 29.007 mgm. and the viable cells 0.003 mgm. per ml. The viability test was performed on the heat-killed part of the vaccine and on the composite vaccine by the technique outlined above.

Vaccinations.

The serological response to each vaccine was investigated on a series of five adult albino rabbits. Prior to vaccination the rabbits were tuberculin tested intradermally with 0.005 mgm. of Purified Protein Derivative (P.P.D.)[x] and the injected areas were examined after 48 hr.

The animals were vaccinated on both sides in the rib area by the scarification method of Rosenthal (53) at a depth of two mm. Four drops of vaccine were spread over each area before puncturing and three drops after the punctures were made.

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[x] Parke, Davis & Co., Detroit, Mich. U.S.A.

Four groups of rabbits were vaccinated with live, irradiated, heat-killed and composite vaccine respectively, using the same technique.

Hyperimmune sera were prepared in rabbits by intravenous injections with heat-killed tubercle bacilli using the method of Rothbard and associates (54). Two rabbits received the highly virulent Ravenel strain of Mycobacterium tuberculosis, var. bovis; two others received the vole bacillus strain, Mycobacterium muris, avirulent in guinea pigs with doses up to 0.1 mgm. The Ravenel strain was grown 10 days in tween-albumin medium and the vole bacillus 14 days in a medium for rapid cultivation of Mycobacterium avium described by Ackart and Murray (1). The cultures were centrifuged, resuspended in physiological saline and heat-killed at 60°C for 45 min. The killed organisms were injected in one ml. amounts twice weekly for four weeks.

Hemagglutination Test.

Phosphate buffered saline solution: sodium chloride 8 gm., sodium monophosphate 3.58 gm., distilled water 1000 ml. The solution was adjusted to pH 7.0 with N hydrochloric acid. The saline was used as the diluent throughout the test.

Preparation of washed, packed sheep's red blood cells: One volume of sheep's blood was collected from the jugular vein into a flask with 1.2 volumes of Alsever's solution (dextrose 2.05 gm., sodium chloride 0.8 gm., sodium citrate 0.42 gm., distilled water 100 ml.). The solution was adjusted

to pH 6.1 with 10% citric acid and sterilized at 110°C for 15 min. The blood was stored at 6°C and was not used earlier than four days after bleeding or later than 45 days. The cells were collected by centrifuging at 2,200 r.p.m. for seven minutes and washed three times with six volumes of the phosphate buffered saline solution. After the third washing the cells were centrifuged 15 min. at the same speed and the supernatant was discarded. The washed, packed red cells were stored at 6°C and used within one day.

Collection and preparation of sera:

Each rabbit was bled from the ear prior to tuberculin testing, and at weekly intervals following vaccination. The cell-free serum was obtained by centrifuging at 2,200 r.p.m. for 10 min. and stored at -20°C from one to four weeks when the test was performed. Prior to absorption the serum, 1:2 dilution, was inactivated in a water-bath at 56°C for 30 min. to destroy the complement. The absorption of non-specific antibodies to constituents of sheep's red blood cells was carried out by the addition of 0.2 ml. packed red cells to two ml. 1:2 dilution heated serum. The serum-cell suspension was held at room temperature 20 min., shaken frequently, and then centrifuged at 2,200 r.p.m. for five min. The process was repeated with another 0.2 ml. packed cells without the removal of the sedimented cells. After the second centrifugation the clear serum was collected and diluted 1:4 in saline. The absorbed serum was stored at 6°C and used within one day.

Sensitization of cells with antigen:

The antigen was a concentrated solution of human type tuberculin, 25% O.T. received from The Animal Diseases Research Institute, Hull, Quebec. Six ml. 1:83 dilution O.T. was used to sensitize 0.1 ml. packed, red blood cells. The sensitization was carried out in a water-bath at 37°C for two hr., with shaking at approximately 15 min. intervals. The cells were then washed three times with six ml. saline and collected by centrifuging at 1,800 r.p.m. for 10 min. The washed, sensitized cells were resuspended in 25 ml. saline, giving a 0.4% concentration. The cells were used the same day on which they were prepared.

Setting up the test:

The absorbed sera were set up in twofold serial dilutions, of 0.4 ml. volumes, ranging from 1:4 to 1:512. The sensitized cells were added in 0.4 ml. volumes to each dilution. Two negative controls one with saline and sensitized cells and a second with a 1:4 dilution of serum and unsensitized cells, were performed with each serum. The Ravenel hyperimmune serum of known titer was included as a positive control each time the test was performed. The sera and controls were incubated in a water-bath at 37°C for two hr., shaken at 20 min. intervals, and held overnight at room temperature.

Reading of the test:

The highest dilution of serum at which definite clumping

occurred was designated the titer, the volume of the sensitized cell suspension added as antigen was not included. The procedure of reading was as follows: Each tube was gently shaken and the pattern of dispersion of the rising cells was noted. The degree of clumping was graded into four groups:-

4 + one or a few large clumps in perfectly clear menstruum;

3 + smaller, sandy-like clumps in clear menstruum;

2 + small clumps in cloudy menstruum;

1 + small, easily dispersible clumps in cloudy menstruum.

In negative tubes the cells rose slowly in a smoke-like manner without any trace of clumping. The end-point titer of a serum was defined as the highest dilution in which 2 + clumps were present; 1 + clumping was recorded but not considered in the evaluation of results.

RESULTS

In series I, five tuberculin negative rabbits were vaccinated with a BCG suspension estimated to contain 10^8-10^9 viable cells per ml. The serological response at weekly intervals was determined by the hemagglutination test (Table V).

The serum titer of the five rabbits before vaccination ranged from 0-1:16; three of the sera were negative and two showed titers of 1:4 and 1:16 respectively. On the other hand, the sera which were obtained after vaccination ranged in titer from 0-1:256; the highest titers over the 13 week period were 1:256 and 1:16 in two rabbits and 1:64 in three. A titer of 1:16 or higher was maintained by four of the five for periods ranging from 7-12 weeks and for three weeks by one. A titer 1:64 was maintained by four rabbits for periods ranging from 1-4 weeks; one animal did not attain this level. When the series was discontinued after the 13th week, four sera showed titers ranging from 0-1:8 and one a titer of 1:16.

A similar pattern of response was demonstrated by three of the rabbits, the serum titer rose slowly, reached a peak around the seventh week and then gradually decreased. A rapid response was noted in one, which showed a prevaccination titer of 1:16 and a titer of 1:256 at the end of the second week. A weak response was in evidence in one of

TABLE V

ANTIBODY RESPONSE IN RABBITS
VACCINATED WITH LIVE BCG

Weeks	Titer as the Reciprocal of Dilution					Mean Titer
	Rabbit 1	2	3	4	5	
0	0	4	16	0	0	0
1	4	8	32	0	4	4
2	4	32	256	4	4	4
3	4	16	128	32	32	32
4	4	16	128	16	32	32
5	8	16	64	64	32	32
6	16	16	64	64	64	64
7	16	32	64	64	64	64
8	16	64	64	32	32	32
9	4	32	32	16	16	16
10	4	16	32	8	16	16
11	4	16	16	4	8	8
12	4	16	16	4	4	4
13	4	16	8	4	0	4

the rabbits, with a maximum titer of 1:16 which occurred around the seventh week.

In this study, the mean titer was taken to be the titer which was reached or exceeded in three or more of the rabbits. On this basis the mean titer before vaccination was zero; at three weeks after vaccination 1:32, at six weeks 1:64 where it remained for two weeks, after which time it fell gradually. The titer was 1:16 or higher for eight weeks. This is shown in Table V.

In series II, five tuberculin negative rabbits were vaccinated with a suspension which was estimated to contain 10^9 bacilli per ml., of which 99.99% were killed by irradiation, and the remaining 0.01% were live cells as determined by the viability test. The data appear in Table VI.

The sera of the five rabbits before vaccination were negative to the hemagglutination test. After vaccination sera ranged in titer from 0-1:256; the highest titer was 1:128 in two rabbits, and 1:256, 1:64, 1:32 in one animal each. A titer of 1:16 or higher was maintained 10-12 weeks in all the animals. A titer of 1:64 was maintained 5-8 weeks by three rabbits, for one week by one and the fifth did not reach this level. When the series was discontinued at 14 weeks the titers ranged from 1:4-1:8.

A typical pattern of serological response to an antigen was shown by two rabbits. An early response was observed in one rabbit which showed a titer of 1:256 at two weeks.

TABLE VI

ANTIBODY RESPONSE IN RABBITS VACCINATED WITH IRRADIATED BCG

Weeks	Titer as the Reciprocal of Dilution					Mean Titer
	Rabbit 6	7	8	9	10	
0	0	0	0	0	0	0
1	8	4	4	4	0	4
2	32	16	8	8	256	16
3	64	16	32	16	256	32
4	64	32	16	16	128	32
5	64	64	16	32	64	64
6	128	32	16	64	32	64
7	64	32	16	64	64	64
8	64	16	16	128	64	64
9	64	64	32	32	32	32
10	64	32	16	64	16	32
11	32	16	16	16	16	16
12	64	16	16	16	8	16
13	32	-	4	16	8	8
14	8	-	4	8	8	8

- test not carried out.

This rabbit maintained a titer 1:64 for seven weeks. One of the rabbits reacted poorly to the vaccine; its highest titer 1:32 occurred twice, both times for one week. A response in the last rabbit appears to have two peaks, one at five, the other at nine weeks. The rabbit was discarded at 12 weeks because it developed a nose infection.

In series II, the mean titer of sera before vaccination was zero. The mean titers after vaccination ranged from 1:4-1:64. A titer of 1:16 or higher appeared at two weeks and was maintained for 11 weeks; a titer 1:32 or higher persisted for eight weeks; a titer of 1:64 was the highest and was maintained for four weeks. At 13 weeks, or the end of the test, the mean titer was 1:8. (Table VI).

In series III, five tuberculin negative rabbits were vaccinated with a suspension of 10^8 heat-killed bacilli per ml. Table VII.

The serum titers of the five rabbits before vaccination ranged from 0-1:4; three of the sera were negative and two showed a titer of 1:4. Serum titers after vaccination ranged from 0-1:64; the highest titers over the experimental period were 1:64 in two rabbits and 1:16 in three. A titer 1:16 or higher was maintained by four of the five rabbits for 3-7 weeks and only for one week by the fifth rabbit. A titer 1:64 appeared in two rabbits at one week and did not occur in the remainder of the rabbits. The series was discontinued at eight weeks when four sera showed titers

TABLE VII

ANTIBODY RESPONSE IN RABBITS VACCINATED WITH HEAT-KILLED BCG

Weeks	Titer as the Reciprocal of Dilution					Mean Titer
	Rabbit:11	12	13	14	15	
0	0	4	0	4	0	0
1	0	8	4	0	8	4
2	4	8	32	8	16	8
3	4	16	32	16	8	16
4	8	16	32	64	16	16
5	16	16	64	32	16	16
6	8	4	32	8	8	8
7	0	0	16	8	4	4
8	0	0	16	4	4	4

0-1:4 and one a titer of 1:16.

A typical pattern of serological response to the vaccine was shown in all the rabbits. The antibody curve in one was low, reaching 1:16 titer at five weeks and maintaining it only for one week.

In series III, the mean titer of sera before vaccination was zero. The mean titers after vaccination ranged from 1:4-1:16. A titer 1:16 appeared at three weeks and was maintained for three weeks; it was the maximum mean titer

observed in the series. At six weeks the mean titer was 1:8; it fell to 1:4 at seven weeks. The test was discontinued at eight weeks.

TABLE VIII

ANTIBODY RESPONSE IN RABBITS VACCINATED WITH COMPOSITE BCG

Weeks	Titer as the Reciprocal of Dilution					Mean Titer
	Rabbit:16	17	18	19	20	
0	0	0	0	0	0	0
1	0	0	0	4	0	0
2	4	0	4	0	4	4
3	8	0	4	4	8	4
4	16	4	8	0	16	8
5	16	8	16	8	32	16
6	8	4	16	4	32	8
7	8	4	8	4	16	8
8	8	4	8	0	16	8
9	8	0	8	0	8	8
10	8	0	4	0	4	4

In series IV, five tuberculin negative rabbits were vaccinated with a suspension which was estimated to contain 10^9 bacilli per ml., of which 99.99% were killed by heat and

the remaining 0.01% were live cells as determined by the viability test. The data are presented in Table VIII.

The sera of the five rabbits before vaccination were negative to the hemagglutination test. After vaccination sera ranged in titer from 0-1:32; the highest titers were 1:8 and 1:16 in two rabbits each and 1:32 in one. A titer 1:16 or higher was maintained by three of the five animals for 2-5 weeks. Two animals did not attain this level. The series was discontinued at eight weeks when four sera showed titers ranging from 0-1:4 and one titer of 1:8.

A typical pattern of serological response to an antigen was shown by three rabbits. Two of the rabbits reacted poorly to the vaccine; the highest titer was 1:8 in each case. The response was at a low level and for a short time.

In series IV, the mean titer of sera before vaccination was zero, and after vaccination ranged from 0-1:16. A titer 1:16 appeared once, at five weeks. At the end of the test, that is at 10 weeks, the mean titer was 1:4. (Table VIII).

Hyperimmune Sera.

For the preparation of hyperimmune sera six rabbits were used; four were vaccinated with the Ravenel strain and two with Mycobacterium muris. The titer estimated after the immunization period was 1:2048 and 1:8192 each in one

of the two rabbits which received the Ravenel strain. Two rabbits which received the vole bacillus showed titers of 1:1024 and 1:4096, respectively. Two of the four rabbits injected with the Ravenel strain died during the third week of the hyperimmunization. Dissection revealed no pathological lesion in either animal.

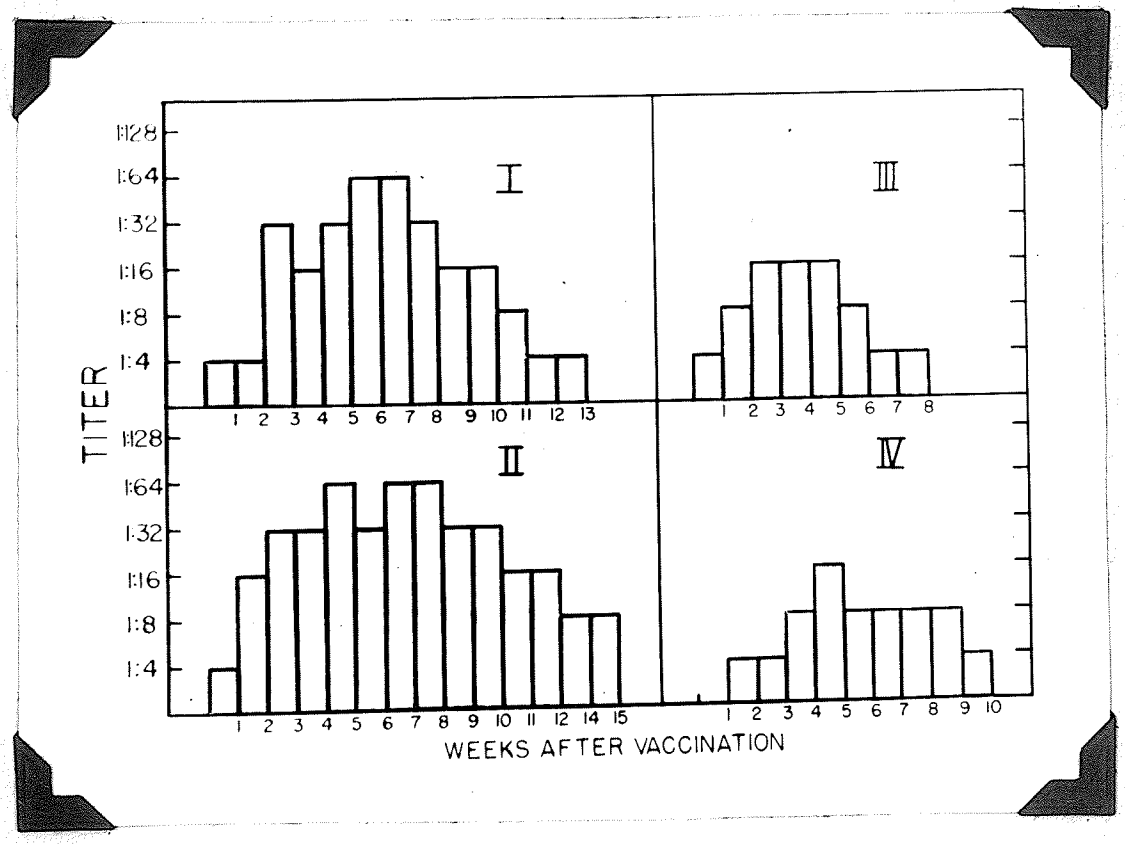

Fig. 1. Mean serological response as measured by hemagglutinin level in rabbits vaccinated with:
I. A live mycobacterial vaccine.
II. An irradiated mycobacterial vaccine.
III. A heat-killed mycobacterial vaccine.
IV. A composite mycobacterial vaccine.

DISCUSSION

The results which are presented demonstrate that hemagglutinins are formed in rabbits' sera in response to vaccination with tubercle bacilli. The response varied in titer and duration with each of the vaccines employed. Accordingly, the evidence suggests that the test may be useful for estimating the degree of serological response and on this basis for evaluating various tuberculosis vaccines.

In this work, as in other biological investigations, there was a difference in the response of individual rabbits in every series. In each series certain rabbits reacted strongly and others weakly as compared to the others in the series. Considering the 20 rabbits in the four series two reacted strongly and five weakly. For this reason the interpretation and evaluation of results are based on the mean response in each series and in special cases reference is made to individual rabbits.

The mean titers observed in series I, where vaccination was performed with living bacilli, are in close agreement with those reported by Hawirko (29). The serological response to the live vaccine was adopted as a standard for the appraisal of titers obtained in the subsequent series.

In series II, the dosage of ultraviolet light employed

produced a vaccine that contained 0.01% viable cells in addition to the large numbers of killed bacilli. The vaccine induced a mean response which was stronger and which was present for a longer period of time than in the standard series (Fig. 1). The difference was shown to be statistically significant according to the "t" test. However, the mean responses in series I and series II, as shown diagramatically in Fig. 1, suggest that the difference between them is small. In addition it may be noted that because the peak titers of individual rabbits in series II occurred at different times the mean response appears to be prolonged and thus the difference between series I and series II is magnified (Tables V and VI). However, the response to the irradiated vaccine, which contained only 0.01% viable cells was certainly on a level with that of the live vaccine (series I). The immunizing power of the irradiated vaccine might be associated either with the cells which were killed by the action of ultraviolet light or with the cells which survived the radiation. Tubercle bacilli killed by ultraviolet light have been shown to possess definite immunizing properties which were as great as those of a living vaccine (43). Other workers (55) also, reported that ultraviolet activity was less destructive to the antigenicity of tubercle bacilli than other lethal agents, such as heat or chemicals. On the other hand Olson et al (41) reported that ultraviolet-killed and heat-killed BCG vaccines were relatively ineffective and were essentially

the same in their protective values. The suggestion that the immunizing power of the vaccine in series II may be due to the presence of living irradiated bacilli is supported by Smithburn and Lavin (59), who effectively immunized rabbits with an irradiated but not killed vaccine. It is difficult to explain how the minute number of surviving cells stimulated a response equal to that of a live vaccine unless the surviving cells possessed superior immunizing properties. An additional possibility which may account for the superior quality of the irradiated vaccine is the combined effect of the irradiated killed and living cells.

The serological response to the heat-killed vaccine (series III, Table VII) was much lower in level and shorter in duration than to the live or to the irradiated vaccine (Fig. 1). There was a significant difference between the serological responses in series III and those in series I or II. One of the main objections to the use of heat in the preparation of vaccines is the denaturizing of proteins. This is suggested by the low response to the heat-killed vaccine even though the thermal factors were kept at a minimum. Another possibility is the loss of antigenicity, as observed by Dubos (20), due to autolytic processes which are favored by certain temperatures. Views on immunization with heat-killed vaccine are divided; some workers (15, 16, 36) maintain that the vaccine confers minimal protection of animals against

an infective dose of tubercle bacilli. On the other hand, other workers (42, 47) claim that the killed vaccine immunizes effectively. The method of administering a heat-killed vaccine influences the serological response considerably. Workers (19, 24) reported that intravenous injection of heat-killed bacilli produces more rapid response and higher titers than the same vaccine when injected subcutaneously or intracutaneously. In this study the intravenous injections of heat-killed bacilli resulted in a high antibody level of from 1:1024 to 1:8192; whereas a titer of 1:256 was the highest in percutaneous vaccination. The death of two of six rabbits during the third week of hyperimmunization suggests that unknown factors, such as hypersensitivity to large amounts of bacillary substance, may have been active.

The composite vaccine of live untreated and heat-killed cells in series IV was used as the control to assess the antigenic power of the irradiated vaccine (series II). The composite vaccine contained the same proportion of living and heat-killed cells as the irradiated living and killed bacilli. The low antibody level in the composite indicates that the 0.01% living bacilli in the vaccine did not intensify the serological response. On the basis of the "t" test there was a significant difference between series IV and I or II, but not between series IV and III.

The results of this study suggest that an irradiated vaccine might be preferred to a living vaccine, since the

wisdom of administering live tuberculosis bacilli may be questioned, and the irradiated vaccine produced an equal or better response than the living vaccine. The serological response to the ultraviolet treated cells, which was much more pronounced than to the heat-killed, indicates that ultraviolet light has less destructive effect on this antigen than has heat. The number of living bacilli in series II and series IV was the same. Likewise, the number of killed bacilli was the same. Consequently, the stronger response in the irradiated vaccine (series II) must have been due to some principle related to irradiation, but not active in heat-killing. If this interpretation is valid, technical refinements such as the number of bacilli, dose of light etc. may improve the irradiated vaccine, which even in the present form has an activity equal at least to that of live BCG. The serological response, both in level and duration, produced by different vaccines may be accepted as indicating that the hemagglutination test offers a means for the study of immunity to tuberculosis.

The presence of tuberculosis bacilli in the body stimulates the production of antibodies, but probably only a few of them protect the host against disease (37). The search for a means of determining and measuring protective antibodies in tuberculosis is continued to the present day. Workers concerned with the question of immunity to the disease have employed a protection test, which measures the survival

time of immunized animals against an infective dose of virulent tubercle bacilli. However, interpretation of this test may be misleading because size of the vaccination dose, physiological state of vaccine, time of injection of the lethal dose and proper species of test animal are unknown. There is divided opinion among workers on the degree of immunity induced by attenuated or by killed tubercle bacilli when the protection test is used as a criterion of the immune state. The hemagglutination test may prove to be helpful in the measurement of immunity, if there could be established a correlation between hemagglutination level and resistance to infection after vaccination. Work along this direction by Gernex-Rieux and Tacquet (25) showed that there was no correlation between titer, as measured by the hemagglutination test, and immunity or cure. Similar observations were made by Raffel (50) who used the precipitin test and did not find any correlation between presence of antibodies and resistance to subsequent infection. It may be noted that the literature dealing with this problem is very limited. However, the hemagglutination test may offer a valuable basis for obtaining additional data on serological response as compared to the limited information provided by the protection test.

In place of guinea pigs, which are widely used for experimental work on tuberculosis, rabbits were used in this study. Resistance to infection with human or bovine tuberculosis bacilli in rabbits is more like that in humans (42)

than the resistance in guinea pigs; and the disease in rabbits more closely resembles that in man. In addition, it is widely accepted that the response to vaccination is greater in rabbits than in guinea pigs and rabbits are more satisfactory for bleeding purposes, especially when weekly serum samples are required.

It may be pointed out, that before vaccination the sera of 20% of apparently healthy rabbits contained hemagglutinins- the sera of three rabbits 1:4 and of one rabbit 1:16. The same evidence has been reported by many workers (29, 32, 54) in connection with human sera. This fact may indicate some kind of immunological over-lapping.

SUMMARY

1. The serological response in four groups of five rabbits vaccinated with live, irradiated, heat-killed and composite BCG vaccines respectively, was measured by the hemagglutination test of Middlebrook and Dubos (39).
2. Hemagglutinins varied in titer and in duration with each of the vaccine 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 the 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.
3. The evidence indicates that the test may be useful for estimating the degree of serological response, and on this basis for evaluating various mycobacterial vaccines.
4. The results indicate that irradiation was effective for the preparation of tuberculosis vaccines, and that heat treatment appreciably reduced the antigenic property of the vaccine.
5. The serological response to a series of intravenous injections of heat-killed bacilli was much higher in titer (1:1024 to 1:8192) than to single percutaneous vaccination performed by the scarification method (0 to 1:250).

6. Before vaccination four of 20 sera from apparently healthy rabbits showed hemagglutinins - three of the sera gave a titer of 1:4, and one a titer of 1:16.
7. The lack of a standardized procedure for the hemagglutination test necessitated a preliminary study, in which the following points were established:
   (a). The Hull O.T. (human) 25% in 1:83 dilution was a more effective sensitizing agent than Lederle O.T. 1:12, Hull O.T. (bovine) 50% in 1:40, or Wellcome O.T. in 1:60 dilution.
   (b). Storage of sera for three months at -20°C did not change the hemagglutinin level; but storage for 18 months lowered it significantly.
   (c). Sera inactivated at 56°C for 30 min. before storage for 14 days and sera inactivated after storage for the same period produced essentially the same titer.
   (d). Phosphate buffered saline as a diluent was more satisfactory than buffered isotonic saline.

REFERENCES

1. Ackart, W.B. and Murray, T.J. A medium for the rapid cultivation of Mycobacterium avium. J. Bact. 62: 75-79. 1951.

2. Anderson, R.J. Licensure of BCG vaccine. Publ. Health Rep. 65: 963-964. 1950.

3. Arloing, S. Agglutination du bacille de la tuberculose vrai. C. R. Acad. Sci. 127: 312. 1898. (cited from ref. 57)

4. Armstrong, A.R. and Orlicki, J. The technique of the sensitized sheep cell haemagglutination test for tuberculosis. Can. J. Med. Techn. 13: 67-75. 1951.

5. Behring, von E. Beitrag zur Frage der Rinder Tuberculose Immunisierung. Beitr. z.exper. Therap. 10: 1-5. 1905.

6. Bezançon, F. and Serbonnes, D. Journ. de physiol. et de pathol. generales. 11: 1097. 1909. (cited from Calmette, A. L'infect. bacil. tuberc. 14th ed. Masson et Cie. Paris. 1936.)

7. Branch, A. and Enders, J.F. The immunization of guinea pigs with heat-killed and formol-killed tubercle bacilli. Am. Rev. Tuberc. 32: 595-600. 1935.

8. Burger, G.N. Bull. Basic Sc. Research. 2: 46. 1928. (cited from ref. 59)

9. Burnet, F.M. Modification of human red cells by virus action. III. A sensitive test for mumps antibody in human serum by the agglutination of human red cells coated with a virus antigen. Brit. J. Exp. Path. 27: 244-247. 1946.

10. Burnet, F.M. and Anderson, S.G. Modification of human red cells by virus action. II. Agglutination of modified human red cells by sera from cases of infectious mononucleosis. Brit. J. Exper. Path. 27: 236-244. 1946.

11. Calmette, A. Sur la vaccination preventive des enfants nouveau-nes contre la tuberculose par le BCG. Ann. Inst. Pasteur. 41: 201-203. 1927.

12. Calmette, A. L'infection bacillaire et la tuberculose chez l'homme et chez les animaux. 14th ed. Masson et Cie. Paris. 1936.

13. Calmette, A. and Massol, L. Sur la precipitation des tuberculines par le serum d'animaux immunises contre la tuberculose. C. R. Acad. des Sc. 149: 760-762. 1909.

14. Calmette, A. Tubercle bacillus infection and tuberculosis in man and animals. 10th ed. Williams and Wilkins Company. Baltimore. 1923.

15. Copper, H.J., Damerow, A.P. and Cohn, M.L. The effect of the inoculation of avirulent tubercle bacilli on subsequent virulent infection in animals. Am. Rev. Tuberc. 33: 721-732. 1936.

16. Damerow, A.P. The immunizing property of heat-killed tubercle bacilli. Am. Rev. Tuberc. 41:512-518. 1940.

17. Dembinski, M. Note sur l'accoutumance des lapins aux doses mortelles de cadavres de bacilles tuberculeux. Compt. rend. Soc. de Biol. 55: 1409-1412. 1903.

18. Deycke, von G. and Much, H. Bakteriolyse von Tuberkelbazillen. München. Med. Wchnschr. 56: 1985-1987. 1909.

19. Dienes, L. and Schönheit, E.W. Resistance to tuberculous infection of guinea pigs rendered skin sensitive with dead tubercle bacilli. Am. Rev. Tuberc. 13: 379-384. 1926.

20. Dubos, R.J. The bacterial cell. 4th ed. Harvard University Press. 1949.

21. Dubos, R.J. and Middlebrook, G. Media for tubercle bacilli. Am. Rev. Tuberc. 56: 334-345. 1947.

22. Elek, S.D. The serodiagnosis of tuberculosis. Proc. Roy. Soc. of Med. 45: 478-480. 1952.

23. Fleming, J.W., Rnyon, E.H. and Cummings, M.M. An evaluation of the hemagglutination test for tuberculosis. Am. J. of Med. 10: 704-710. 1951.

24. Freund, J. and Opie, E.L. Sensitization and antibody formation with increased resistance to tuberculous infection induced by heat-killed tubercle bacilli. J. Exp. Med. 68: 273-298. 1938.

25. Gernex-Rieux, C. and Tacquet, A. Les reactions d'hemagglutination et d'hemolyse conditionnee dans la tuberculose. Ann. Ins. Pasteur, Lille. 4: 17-104. 1951.

26. Grancher, J. and Martin, H. Etude sur la vaccination tuberculeuse. Rev. de la tuberc. 4: 289. 1893. (cited from J.A. Myers. The ever-continuing search for immunity in tuberculosis. Postgrad. Med. 12: 3. 1952.)

27. Haley, R.R., Davey, W.N., Adcock, J. and Owen, C.R. Hemagglutination reaction following BCG vaccination in human subjects. Am. Rev. Tuberc. 66: 58-62. 1952.

28. Hall, W.H. Antibodies in tuberculosis. Bull. U. Minnesota Hosp. and Minn. Med. Foundation. 22: 557-567. 1951.

29. Hawirko, R.Z. The hemagglutination reaction to BCG and vole bacillus vaccination. Can. J. Microb. 1: 79-84. 1954.

30. Hilson, T.R.F. and Elek, S.D. The hemagglutination reaction in tuberculosis. J. Clin. Path. 4: 158-164. 1951.

31. Hollander, A.G., Frobisher, M. Jr. and Kalish, K. Clinical evaluation of the hemagglutination reaction. Am. Rev. Tuberc. 67: 497-502. 1953.

32. Kirby, W.M.M., Burnell, J.M. and O'Leary, B. Evaluation of the hemagglutination reaction in the diagnosis of active tuberculosis. Am. Rev. Tuberc. 64: 71-76. 1951.

33. Klimmer, T. Das Dresdner Verfahren, Rinder mit Hilfe nicht infectioser Impfstoffe gegen die Tuberkulose zu immunisieren. Ztschr. f. Tiermed. 12: 81-87. 1908.

34. Koch, R. Ber. klin. Wchnschr. 19: 221. 1882. (cited from Jordan, E.O. and Burrows, W. Textbook of bacteriology. W.B. Saunders Company, Philadelphia, 1948.)

35. Levy, E., Blumenthal, F. and Marxner, A. Experimentelle Untersuchungen über Tuberkulose. Centralbl. f. Bakt. 46: 278-282. 1908.

36. Liebow, A.A., Burn, C.G. and Soper, W.B. A comparison of the effects of BCG and of heat-killed organisms on the course of a subsequent infection with virulent tubercle bacilli in the guinea pig. Am. Rev. Tuberc. 41: 592-604. 1940.

37. Maher-Loughnan, G.P. and Hilson, G.R.F. The haemagglutination reaction in relation to the management of pulmonary tuberculosis. Tubercle. 33: 297-303. 1952.

38. Melvin, I., Klein, G.C., Jones W. and Cummings, M.M. An evaluation of media for diagnostic cultures of tubercle bacilli. Am. Rev. Tuberc. 63: 459-463. 1951.

39. Middlebrook, G. and Dubos, R.J. Specific serum agglutination of erythrocytes sensitized with extracts of tubercle bacilli. J. Exp. Med. 88: 521-528. 1948.

40. Mollow, M. and Kott, T.J. A comparative study of the hemagglutination test for antibodies and its hemolytic modification in tuberculosis. Am. Rev. Tuberc. 65: 194-199. 1952.

41. Olson, B.J., Habel, K. and Piggott, W.R. A comparative study of live and killed vaccines in experimental tuberculosis. United States Pub. Health Reports. 62: 293-302. 1947.

42. Opie, E.L. and Freund, J. An experimental study of protective inoculation with heat killed tubercle bacilli. J. Exp. Med. 66: 761-788. 1937.

43. Paterson, J.C., Crombie, D.W. and Coles, J.C. Protection by killed vole bacillus vaccine against experimental tuberculosis in guinea pigs. Can. J. Research. 27: 37-42. 1949.

44. Pearson, L. and Gilliland, S.H. Some experiments upon the immunization of cattle against tuberculosis. J. Comp. M. Vet. Arch. 23: 673-680. 1902.

45. Petroff, S.A., Branch, A. and Jennings, F.B. Jr. Resistance of animals sensitized with heat-killed tubercle bacilli to a measuring infecting dose. J. of Immunol. 16: 233-256. 1929.

46. Petroff, A.S., Branch, A. and Steenken, W. Jr. Microbic dissociation. III. B.C.G. (Bacillus-Calmette-Guerin). Proc. Soc. Exper. Biol. and Med. 25: 14-18. 1927-1928.

47. Petroff, S.A. and Steenken, W. Jr. Resistance of guinea pigs vaccinated with Bacillus Calmette-Guerin (B.C.G.). J. of Immunology. 19: 79-92. 1930.

48. Porter, J. J. Infect. Diseases. 7: 87. 1910. (cited from Calmette, A. L'infect. bacil. tuberc. 14th ed. Masson et Cie. Paris. 1936.)

49. Potter, T.S. Use of bacterial asphyxia for homologous antigen production in experimental immunization against human-type tuberculosis. Proc. Soc. Exper. Biol. and Med. 54: 143-145. 1943.

50. Raffel, S. The relationship of acquired resistance, allergy, antibodies and tissue reactivities to the components of the tubercle bacillus. Am. Rev. Tuberc. 54: 564-573. 1946.

51. Raw, N. A tuberculosis immunizing vaccine. Brit. M. J. 1: 594-596. 1921.

52. Roberts, E.C. and Jones, L.R. Detection of minute amounts of serum antibody agglutination of antigen-coated bacterial cells. Proc. Soc. Exp. Biol. and Med. 47: 11-14. 1941.

53. Rosenthal, S.R. The multiple puncture method of BCG vaccination. Am. Rev. Tuberc. 39: 128-134. 1939.

54. Rothbard, S., Dooneief, A.S. and Hite, K.E. Practical application of a hemagglutination reaction in tuberculosis. Proc. Soc. Exper. Biol. and Med. 74: 72-75. 1950.

55. Sarber, R.W., Nungester, W.J. and Stimpert, F.D. Immunization studies with irradiated tuberculosis vaccines. Am. Rev. Tuberc. 62: 418-427. 1950.

56. Scott, N.B. and Smith, D.T. A simple modification of the Middlebrook and Dubos hemagglutination test for serum antibodies to products of tubercle bacilli. J. Lab. and Clin. Med. 35: 303-307. 1950.

57. Simintzis, G. and Sohier, R. La sero-agglutination de S. Arloing et P. Courmont dans le diagnostic de la tuberculose bovine. Bull. l'Acad. Vet. France. 23: 385-391. 1950.

58. Smith, D.T. and Scott, N.B. Clinical interpretation of the Middlebrook-Dubos hemagglutination test. Am. Rev. Tuberc. 62: 121-127. 1950.

59. Smithburn, K.C. and Lavin, G.I. The effect of ultraviolet radiation on tubercle bacilli. Am. Rev. Tuberc. 39: 782-793. 1939.

60. Suter, W.E. and Dubos, R.J. Variability of BCG strains. J. Exper. Med. 93: 559-561. 1951.

61. Suter, W.E., Schaefer, W.B. and Dubos, R.J. Heterogeneity of BCG strains. Proc. Soc. Am. Bact. 1951, page 90.

62. Wadsworth, A., Maltaner, F. and Maltaner, E. A study of the complement fixation reaction in tuberculosis. J. Immunology. 10: 241-331. 1925.

63. Wassermann, A. Ueber experimentelle Grundlagen für eine spezifische Serodiagnostik auf aktive Tuberkulose. Deutsche Med. Wochnschr. 49: 303-308. 1923.

64. Webb, G.B. and Williams, W.W. Immunity in tuberculosis: A further report on its production by the inoculation of increasing numbers of bacilli. J. Med. Research. 24: 1-4. 1911.