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VACCINATION AGAINST TUBERCULOSIS WITH ULTRASONIC-VIBRATED TUBERCLE BACILLI

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Numerous attempts have been made by many investigators[1-3] to induce protective immunity against tuberculosis by a variety of vaccination programs. Early studies were initiated shortly after the discovery of the tubercle bacilli by Koch in 1882. Since this time, the majority of experiments have been conducted on the basis of four types of vaccines as follows:

1. Preparations of tubercle bacilli killed by a variety of physical and chemical methods.
2. small numbers of living, virulent tubercle bacilli.
3. species of *Mycobacterium* isolated from nonmammalian sources.
4. avirulent organisms of originally virulent strains which were attenuated by various methods.

Immunization with a variety of living, attenuated strains and with several non-living preparations has been shown to produce a significant degree of enhanced resistance for both domestic and laboratory animals as well as man. However, only one of these has been used to any extent in the latter. This vaccine is prepared from an attenuated bovine culture and is known as BCG (Bacille Calmette-Guérin).

Vaccination with BCG preparations has stimulated numerous controversies during the past four decades. The general desirability of this approach to the tuberculosis problem has been evaluated on the basis of efficacy, safety etc. Millions of people have been vaccinated with BCG vaccines during recent years but there is still no absolute agreement on this subject. As a result, there are persuasive arguments for and against the use of these vaccines in the current journals.

The literaure[4-7] contains numerous reports on the advantages and disadvantages of BCG vaccines. The data on this subject can be summarized as follows:

1. The vaccines give some protection in those individuals who are directly exposed to tuberculosis infection either at home or at work.
2. a reduction of tuberculosis occurs in many of the general populations wherein the individuals have low living standards and high tuberculosis rates.
3. the extent and duration of protection, as well as the contribution to the control of tuberculosis in areas where tuberculosis services are good, should still be regarded as problematical.

Ultrasonic-vibrations were used by Kress[8] in 1948 to kill a suspension of virulent human tubercle bacilli. A few guinea pigs were vaccinated subcutaneously with the killed suspension and challenged six weeks later by the intranasal introduction of

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virulent tubercle bacilli. The animals failed to develop tuberculosis but it should be emphasized that the experiment lacked adequate controls. Therefore, this study cannot be considered to have much significance.

Our objective was to investigate the immunizing properties of ultrasonic-treated tubercle bacilli and to compare these preparations with BCG vaccines. We were especially interested in this approach since chemically and heat-treated whole-cell vaccines have been studied for years with questionable promise. It was decided to use several strains of tubercle bacilli which were both virulent and avirulent for man.

MATERIALS AND METHODS

The vaccines reported in this study were prepared using *Mycobacterium tuberculosis* varieties identified as H37, H37Rv, H37Ra as well as a chromogenic strain of acid-fast bacillus. Suspensions of each organism were vibrated separately at 10,000 oscillations* per minute for periods of 5-15 minutes. Since viable organisms were found in all treated preparations it was decided to treat all preparations with 0.35 per cent tricresol in order to inactivate the small number of living tubercle bacilli and have it act as a preservative at the same time. Multiple subcultures for sterility testing were examined for 150 days because it has been our experience that 80-120 days was not sufficient. A total of eleven vaccines (see Table I) were prepared using the above methods in an attempt to obtain preparations which might be effective immunizing agents.

Table I

ULTRASONIC TREATMENT OF TUBERCLE BACILLI

Vaccine #	Organism	Ultrasonic Vibrations in Minutes	mg of Nitrogen per 100 ml of specimen
1	H37Rv	5	1108
2	H37Rv	10	1198
3	H37Rv	10	1007
4	H37Rv	15	1108
5	H37Rv	15 *supernatant	33
6	H37	5	780
7	H37	10	697
8	H37	15	617
9	H37	15 *supernatant	32.8
10	H37Ra	15	71.8
11	Chromogen	15	61.5

*The supernatant of each of the 15 minute treated H37Rv (#5) and H37 (#9) were also tested for their immunizing properties.

This report deals with two series of experiments. The first consisted of an evaluation of the evelen vaccines using a group of 10 guinea pigs for each preparation. The second series consisted of two ultrasonic-treated antigens as well as BCG. Guinea pigs weighing

*A sonic oscillator, manufactured by Raytheon Manufacturing Company, Waltham, Mass., was used.

approximately 300 grams were used throughout these studies. For randomization, the cages in any one experimental group were distributed so that one cage would be found in the first quarter of the total number, a second cage in the second quarter of the total and so on. Tuberculin sensitivity testing of control and vaccinated animals was conducted using Eli Lily's tuberculin preparation. The tuberculin tests were performed 28-30 days after the initial vaccination procedure together with tests of the unvaccinated controls. The results were read at 24 and 48 hours, a positive reaction being 5 mm. or more in minimum diameter. In some instances, reactions were strong with a blanched, later necrotic, center.

The immunization doses were administered subcutaneously in the inguinal region. Each animal received five injections at 7 day intervals with the following volumes: 0.5, 1.0, 1.5, 2.0 and 2.0 mls. A comparable group of animals recieved corresponding volumes of saline. A supply of freeze-dried vaccine was obtained from Sol Rosenthal of the Research Foundation, Chicago. It was administered by the "Multiple Puncture Disc Technique" as outlined in the directions of the supplier. Three weeks after the last immunizing dose the animals were challenged with 1 ml. of a virulent strain of *Mycobacterium tuberculosis* var. *hominis* (approximately 0.1 mg., semi-dry weight, of a ten day culture) subcutaneously in the inguinal area.

The experiments were evaluated in two ways: (1) the extent of gross and histological tuberculous infection (Table 2, 3) as measured by an adaptation of the Feldman Index[9]. Observations were as detailed as possible on all major organs — lungs, liver, spleen, inguinal and retroperitoneal nodes. Acid-fast smears and cultures were also performed on the specimens. (2) by the average survival time for each group of animals.

Table II*

Key Table for Evaluating *Histologically* the Extent and Character of the Tuberculous Lesions in Experimentally Infected Guinea Pigs. Assign the Highest Number Corresponding to the Description of the Lesion in Each Tissue.

Extent and Character of Lesions in Section Examined	Spleen	Lung	Liver	Site of Inoculation and Contiguous Lymph Nodes	If Lung is Negative Examine Tracheo-bronchial Lymph Nodes
Progressive lesions present					
Extensive involvement	35	30	25	10	10
Moderate	20	20	20	10	10
Slight	10	10	10	10	10
Nonprogressive lesions only					
Fibrosis, hard tubercles	3	3	2	1	1
Fibrosis or calcification only	1	1	1	1	1
Maximal values = 100 total	35	30	25	10	10 (If lung is negative)
If only inactive lesions, maximal values less than 10	3	3	2	1	1 (If lung is negative)

*This table was copied from Feldman's paper (9).

Table III

Method for Evaluating by *Gross Appearance* the Extent and Character of Tuberculous Lesions in Experimentally Infected Guinea Pigs*.

Extent of Involvement in Tissues Examined	Spleen	Lung	Liver	Site of Inoculation	Tracheal bronchial Nodes with Negative Lung
Extensive 3+ to 4+	35	30	25	10	10
Moderate 2+	20	20	20	10	10
Slight 1+	10	10	10	10	10
Maximum Values 100 Total	35	30	25	10	10 (If lung is negative)

*Slight Modification of Feldman's procedure[9].

Result: The purpose of the first series of experiments was to ascertain whether there was any evidence that the ultrasonic-treated organisms possessed potential antigenic value. The animals in the eleven experimental and one control group — No. 12 — (Table 4) were autopsied at the time of death and the average survival rates are summarized in Table 4. The data show that antigens #7 and #8 increased the survival time to a greater extent than the other antigens. Statistical evaluation of the results obtained on the animals in each group showed that the probabiliy values for vaccine #7 and #8 were significant.

Table IV

Identification of Vaccines

	H37Rv				H37					H37Ra	Chromo-genic	Control
	1	2	3	4	5	6	7	8	9	10	11	12
Average number of days after challenging	68	69	62	81	36	85	128	108	40	86	77	48

The apparent increase in resistance to tuberculosis produced by antigens #7 and #8 in the first series of experiments led us to compare their immunizing properties with those of BCG as well as to confirm the results of the first series of experiments. Thirty animals were used in each group of the second experimental series including a similar number in two controls (one of which received saline and the other no injections). The guinea pigs were tested with tuberculin after the 5 immunization doses had been given at weekly intervals. Slightly more than fifty per cent of the animals showed strongly positive tuberculin tests. The other fifty per cent were either weekly reactive or negative.

The results of the survival times for the animals of the second experimental series are listed in Table 5. The data serve to emphasize that BCG produced much greater resistance against tuberculosis than did vaccine preparations #7 and #8. Thus, there is a striking degree of protection (Table 5) exhibited by BCG vaccine. This preparation produced a survival time of greater than 107 days as compared to the 41, 39, 51 days for vaccines #7, 8 and the saline controls respectively. The control group which did not receive a challenge dose (Table 5) was included to evaluate intercurrent infections in the animal colony. The animals which survived in this

latter group were sacrificed 200 days after the date of challenge and showed an average survival value of greater than 161 days.

Table V

Results of Experimental Series #2

Vaccine	Description	Per cent of animals Tuberculin Positive after Immunization	Average Survival Period in Days
#7	H37 10 min.	55%	41
#8	H37 15 min.	66%	39
BCG	Research Foundation	59%	>107
Controls	Saline —Animals challenged	0%	51
Controls	—Saline only —No challenging dose	0%	>161

All animals which had received a challenging dose and survived for a two week period thus avoiding intercurrent infection, eventually showed moderate to extensive tuberculosis. This conclusion has been based on the results obtained with the Feldman method[9] for evaluating both the gross and histological tuberculosis (Tables 2 and 3) changes in the infected guinea pigs.

Intercurrent infections were usually high in all groups. Therefore, it was always necessary to quarantine the animals for several weeks before beginning the immunizing dose. To avoid errors due to intercurrent disease it was imperative that all animals be autopsied rather than to depend on death and survival rates only.

DISCUSSION

The results of the first experimental series provides further evidence (Table 4) that a degree of enhanced resistance to tuberculosis can be produced not only by living but also by killed tubercle bacilli. Electron micrographs of the ultrasonic treated suspensions showed both intact and fragmented cells. Vaccines number 5 and 9 which consisted of supernatant from H37 preparations were immunogenically inactive. Thus, one may conclude that the sediments were capable of enhancing resistance to tuberculosis to a greater extent than the supernatant materials under test.

An important but controversial issue in tuberculosis is the role of tuberculin skin hypersensitivity in protective immunity. The experiments in which the vaccines were capable of increasing the resistance of the animals to tuberculosis often did so without making the animals skin-hypersensitive to tuberculin. The reverse was also common — the tuberculin positive animals frequently showed little or no resistance to the challenging dose of tubercle bacilli. Emphasis should be placed on the fact that the absence of skin hypersensitivity to an antigen does not always indicate total absence of systemic hypersensitivity. Furthermore, tuberculin preparations which were employed to test for sensitivity consisted of the usual heated preparations, and the absence of a reaction to these does not necessarily mean that the animal would not be sensitive to unheated tuberculoproteins. Thus, the independence of tuberculin skin hypersensitivity and acquired resistance to tuberculosis should be considered in discussing this complex subject.

The work of Weiss[3] as well as this report support the contentions of other investigators that the development of acquired immunity to tuberculosis does not require infection with living tubercle bacilli. Immunity to tuberculosis can be bestowed by either components or whole nonliving cells which at times may also lack skin-sensitizing properties.

An attempt was made with the first series of our experiments to determine the extent of antigenic activity of the eleven vaccines. Having demonstrated that vaccines # 7 and 8 were statistically significant when compared with the controls, the second series was designed to answer two questions:

1. Would the vaccines retain their antigenic activity on storage for a one year period?
2. How effective would the preparations be when compared with BCG?

A comparison of the survival periods (Table 6) shows vaccines #7 and 8 to be of extremely short durations. In fact, the figures are smaller than those shown for the control group. This might indicate that toxic substances as well as other unknown factors may have contributed to a shortening of the survival time. The wide differences between the results of the first and second series may possibly be due to loss of potency during the storage period.

If one examines the survival period for the BCG group (107 days) in series number 2 (Table 6) and compares this with the results of series number 1 (128 and 108 days), the data show that the nonliving vaccines were at least as effective against tuberculosis as the living one (BCG). It is evident from a perusal of the literature that the majority of experimental data in which nonliving antituberculosis vaccines (ie. phenol killed, methanol extracts etc.) were tested that such preparations possess approximately equal immunogenicity as living, attenuated ones. Although a number of technical problems — variation in dosage, route of administration, vaccination-challenge interval, method of evaluating the data, etc. — require further attention. There is sufficient evidence that killed tubercle bacilli and some of their extracts definitely have immunizing properties[12].

Table VI

Survival Period in Days for the Two Series of Experiments

	Series 1	Series 2
Vaccine #7	128	41
Vaccine #8	108	39
Control	48	51
Saline + Challenging dose		
BCG	not done	107

Safety of vaccination is another very important consideration since one wants to prevent, not initiate progressive disease. It should be understood that safety, as it refers to any pathologic process due to vaccination, is not always assumed. Therefore,

it is of primary importance that the toxigenic properties of nonliving vaccines be investigated.

It is possible that there may be a decrease in efficacy with corresponding increases in dosages[13] even to levels still below that at which physical symptoms of illness appear. This is an indication that a threshold area of toxicogenicity exists in evaluating "tuberculosis vaccines".

Other arguments in favour of killed vaccines have been previously reviewed at length by Weiss[12]. The results of the first experiment in this report are an indication that ultrasonic-treated bacilli have immunizing properties. The increase in resistance demonstrated by the prolonged survival time of the animals suggests that further studies be done. Furthermore, the data obtained in the second experiment show BCG to be a superior vaccine. One cannot discount the fact that vaccines #7 and 8 showed comparable effectiveness in experimental series number one.

SUMMARY AND CONCLUSION

Two of eleven vaccines prepared by ultrasonic vibration of four strains of *Mycobacterium* were found to stimulate increased resistance to tuberculosis when compared to the saline control group which was challenged with comparable inoculum. The data were statistically significant .The other nine preparations did not show protective activity to the same extent.

The two vaccines were not as effective after storing at 4°C for a one year period. In fact the 'stored' vaccines appeared to cause a toxic effect. Parallel experiments showed that BCG prolonged the survival time to a much greater degree than the two 'stored' vaccines.

A review of the data suggests that the ultrasonic-treated antigens (vaccine #7, 8) in the first series of experiments were equally as potent as BCG in the second series. For this reason as well as the fact that the results of the first experimental series were statistically significant, further studies should be undertaken using freshly prepared ultrasonic treated vaccines and BCG.

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