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Pathogenicity of the Tubercle Bacillus.*

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The factors responsible for the ability of *Mycobacterium tuberculosis* to produce disease in a susceptible host have long been enigmatic. The criteria commonly employed to judge the probable virulence of many other pathogenic bacteria have not been applicable in this instance. For example, although dissociation occurs in cultures of tubercle bacilli,[1] smoothness or roughness of colonial form does not provide a satisfactory basis for predicting the activity of this organism in the tissues of a susceptible species of host.[2] It is mystifying indeed that an attenuated bacterium such as the B.C.G. (Bacillus Calmette-Guérin) should possess, so far as we know, all the properties of the virulent tubercle bacillus except the ability to produce a progressive generalized infection. At the site of injection it induces the typical tuberculous lesion, and from this area viable bacilli may be cultured after many months.

It is difficult to deduce from available information whether the virulent tubercle bacillus possesses some cytoplasmic constituent analogous to the somatic antigens associated with virulence in many other bacterial species. One of the major functions of such substances in permitting organisms to multiply in tissues is the phagocyte-repellent effect. No such effect is apparent in the case of *M. tuberculosis,* for virulent as well as attenuated organisms are readily taken up by monocytic phagocytes. Its pathogenicity may conceivably depend upon a metabolic feature of the bacterial cell, the virulent organism being better able to propagate in the environment supplied by viable tissues. For this view also evidence is lacking.

Should virulence depend in part upon a specific cellular antigen, it seems a rational probability that the acquisition of immunity will also be related to this substance, for in the final analysis acquired resistance means the ability of the body specifically to "neutralize" the virulence mechanism of the bacterium. A familiar case in point is the protection provided by the antibody directed against the capsular S.S.S. of *D. pneumoniae.* If, on the other hand, virulent tubercle bacilli are distinguished by a metabolic trait which favors their growth in the tissues, then it is quite possible that no ordinary immunologic device with which we are familiar will divulge the nature of acquired resistance to them.

Several years ago we undertook a study designed to reveal whether some one antigenic component of *M. tuberculosis* may be responsible for inducing acquired resistance, and as a corollary, whether this factor would enable us to demonstrate, on an immunologic basis, the dissociation or identity of resistance and allergy in this disease.[3]

Attempts to induce the resistant state in the absence of allergy have so far been unsuccessful. A good deal of attention has been devoted to the polysaccharide of the bacillus in this connection. This substance however is haptenic, and its reconversion to a complete antigen has not proved satisfactory, so that a fair test of its immunizing ability has not been possible. However, we have found that when this substance was injected daily in amounts up to 15 mg to guinea pigs throughout a 10-week course of immunization with B.C.G., and then for 20

* Carried out with the aid of grants from the California Tuberculosis and Health Association and the National Tuberculosis Association.

† With the assistance of Dr. Carl M. Johnson, Mrs. Jessie Billon and Mr. Waldo H. Hanns.

1 Petroff, S. A., and Steenken, W., Jr., *J. Exp. Med.,* 1930, **51**, 831.

2 Smithburn, K. C., *Am. Rev. Tuberc.,* 1937, **36**, 637.

3 Raffel, S., *Stanford Med. Bull.,* 1943, **1**, 209.

weeks subsequent to infection with virulent organisms ($H_{37}Rv$), it failed to antagonize the acquired resistance produced by the B.C.G.[4] It appears then that acquired immunity to *M. tuberculosis* is not related in any major degree to an antibody type of response to this particular bacillary antigen. The purified phosphatide, the wax, and the protein have also been employed as vaccinating materials, and these have failed to induce resistance to the bacillus of origin.[5]

More success has attended our studies of the possible relationship of tuberculous allergy to resistance. It has been appreciated for many years that this hypersensitive state is a response to the protein of the organism. Peculiarly enough, however, the tuberculin-type of allergy cannot be initiated by the use of the bacillary protein in isolated form, although the substance has marked antigenic properties and on injection into normal animals regularly induces humoral antibodies and the anaphylactic state. We have found that the delayed type of tuberculin hypersensitivity can be established in the absence of tubercle bacilli by employing the chloroform extractable "wax" of the bacillus. Such wax contains a small proportion of protein, and when injected into normal guinea pigs it regularly establishes good levels of delayed reactivity to Old Tuberculin.[5] The application of several criteria for the tuberculin-type of hypersensitivity, including vulnerability of such sensitized tissues to tuberculin in tissue culture, and failure to transfer the sensitivity passively via serum, have demonstrated that this is true tuberculous allergy. Tests on a limited number of guinea pigs have indicated that animals rendered hypersensitive by this means do not possess acquired resistance to tuberculous infection. This observation has convinced us that allergy and acquired immunity are immunologically distinguishable.

Experimental. Our more recent efforts to isolate a resistance-inducing factor from the bacillus has led us to investigate antigens of the "Boivin type."[6] These are complex substances composed of polysaccharide, lipid and protein, and have been obtained from a variety of the Gram-negative enteric bacilli. They are of utmost immunological importance in such organisms, since they comprise the essential immunizing antigen and also reveal the toxic properties commonly associated with these bacteria. Choucroun[7,8] has reported the presence of a toxic fraction in paraffin oil extracts of tubercle bacilli which apparently induces acquired resistance. This is a polysaccharide ester of mycolic acid, and may be related to the Boivin type of antigen.

Since Boivin's original demonstration of the extractability of antigenic complexes by trichloracetic acid,[6] several other methods have been described for accomplishing this end. Because we had no foreknowledge of the relative susceptibility of the tubercle bacillus to these extraction methods, we decided to employ them all on a comparative basis. The procedure of Raistrick and Topley[9] makes use of trypsin digestion; Morgan[10] employed diethylene glycol, and Walker[11] used urea for this purpose.

Preparation of Complexes. Human $H_{37}Rv$ bacilli were cultured on a modified Long-Seibert synthetic medium. At the end of 3 weeks the organisms were separated from the medium and washed with distilled water. A portion of these bacilli was washed in 50%, and then in absolute acetone in a refrigerated centrifuge. Immediately afterward the bacilli were dried in a vacuum dessicator over sulphuric acid. The remainder were employed in the fresh moist state. This difference in treatment was instigated by uncertainty as to the effect of even a short period of acetone treatment upon the organisms in view of the presence of acetone soluble fats in tubercle bacilli. Batches of the cells were extracted as shown in Table I, where the

[4] Unpublished observations.

[5] Raffel, S., *Am. Rev. Tuberc.*, 1946, **54**, 574.

[6] Boivin, A., Mesrobeanu, I., Mesrobeanu, L., and Nestorescu, B., *C. R. Soc. Biol.*, 1934, **115**, 306.

[7] Choucroun, N., *Science*, 1943, **98**, 327.

[8] Choucroun, N., *Science*, 1947, **105**, 46.

[9] Raistrick, H., and Topley, W. W. C., *Brit. J. Exp. Path.*, 1934, **15**, 113.

[10] Morgan, W. T. J., *Biochem. J.*, 1937, **31**, 2003.

[11] Walker, J., *Biochem. J.*, 1940, **34**, 325.

TABLE I.
Essential Information Concerning the Extraction of Complexes from Tubercle Bacilli.

Bacillary batch No.	Treatment prior to extraction	Grams of bacilli extracted	Extraction method	Mg of lyophilized complex obtained
1	Washed with water, acetone dried	15.6 (dry wt)	Boivin—trichloracetic acid	219.0
2	Washed with water	10 (moist wt)	" "	50.0
3	Washed with water, acetone dried	15.9 (dry wt)	Raistrick and Topley—trypsin digestion	257.0
4	Washed with water	25.2 (moist wt)	Walker—urea	51.4
5	Washed with water, acetone dried	15.4 (dry wt)	Morgan—diethylene glycol	26.5

yields of the individual complexes are also indicated. All of the complexes were lyophilized and stored in a carbon dioxide box. They formed colloidal suspensions when dissolved in saline or water.

During the course of their preparation these substances were subjected to the following physical manipulations calculated to free the extracts of intact bacilli:

The 2 trichloracetic acid preparations were freed of most bacteria by centrifugation in the cold. The supernatants were then ultracentrifuged for 15 minutes at 12,000 r.p.m. Following this the extracts were passed through Jena 5/3 glass filters, and were then lyophilized. The trypsin and urea preparations were freed of most of the organisms by Sharples centrifugation. These fluids were filtered through Chamberland L_3 candles prior to lyophilization.

The diethylene glycol extract, because of its smaller volume and the nature of the fluid, was spun in the ultracentrifuge at 25,000 r.p.m. for one hour. This material was subsequently filtered through a Chamberland L_3 candle also.

Treatment of guinea pigs. These lyophilized preparations were freshly dissolved for each immunizing injection. Fifty-three guinea pigs weighing between 400 and 500 g, and negative in skin test to Old Tuberculin 1:10, were given the first injections of these complexes on August 19, 1946. Ten or 11 animals were apportioned to each of the preparations. These groups of guinea pigs will be referred to by the numbers used to designate the bacillary batches in Table I. The dose of each complex administered was 0.5 mg in 0.25 cc of saline, injected subcutaneously. Three weeks later skin tests with O.T. 1:10 proved negative in all groups. One month following the first injection, second injections of the same dose of the respective complexes were administered, this time intracutaneously. This was repeated a month later, subcutaneously. Thus, during a period of 8 weeks these animals received 3 doses of complex, totaling 1.5 mg for each animal.

Twelve weeks after the first injection of each complex, routine skin tests were carried out with undiluted O.T. and the results were quite unexpected. Every one of the 43 animals comprising Groups 1, 2, 3 and 4 developed exceedingly large necrotizing reactions, averaging 29 mm in diameter. These were unusually severe in that practically the entire areas were necrotic within 24 hours. One week later tests were repeated in 12 of these guinea pigs with less concentrated O.T. (1:10). The average diameter of reaction was 25 mm, and in 10 of these necrosis occurred. None of the animals receiving the diethylene glycol complex developed sensitivity to O.T., either at this time nor during several succeeding months of injections.

One of the animals of Group 4 (urea complex) which died as a result of the initial tuberculin reaction, showed marked tuberculosis involving the axillary lymph nodes and the spleen. Histologic examination revealed lesions with bacilli in these locations. All of the remaining animals in Groups 1,

TABLE II.
Gross Tuberculosis in Regional Lymph Nodes, Abdominal and Thoracic Viscera of Guinea Pigs Receiving Injections of Complexes.

Guinea pig group	No. of pigs in groups	No. showing involvement of lymph nodes regional to inject. (axillary and ing.)	No. with involvement of liver and/or spleen	No. with involvement of lungs and/or tracheobronchial lymph nodes
Trichlor. extract of dried bacilli	9	8	9	7
Trichlor. extr. of fresh bacilli	9	8	8	6
Trypsin extr. dried bacilli	11	8	6	4
Urea extr. fresh bacilli	8	7	7	5
Total	37	31	30	22

2, 3 and 4 were subsequently examined at autopsy for the degree and extent of dissemination of tuberculosis. The overall results are shown in Table II. Thirty-one of the 37 animals showed gross evidence of infection at 3 months or later following the first injection of the respective complexes. The animals which failed to reveal such evidence had previously responded to Old Tuberculin with skin reactions equal in severity to those seen in the pigs with obvious disease. Of the 31 animals, most showed advanced infection, and a few were striking in this regard. As indicated in Table II infection was most evident in the abdominal viscera and in the axillary and inguinal lymph nodes draining the areas of injections of the complexes, while a smaller proportion of animals showed disease also in the lungs or tracheobronchial lymph nodes. In no case was there infection in the lungs or tracheobronchial nodes in the absence of abdominal visceral or axillary-inguinal node involvement. It is thus rather apparent that infection was consequent to the injections, and was not spontaneous as the result of aspiration of bacilli from the environment. Added to this evidence is the fact that groups of normal unused guinea pigs as well as those receiving other, noninfectious vaccinating treatments such as the diethylene glycol complex and various preparations of proteins derived from the tubercle bacillus, were housed in the same large room during the entire period of this experiment. These have never responded to repeated tests with concentrated Old Tuberculin, nor has evidence of tuberculosis been encountered in autopsies of many such animals. Furthermore, it has been a general experience as well as our own that guinea pigs are very unliable to spontaneous infection with *M. tuberculosis* even after intimate and prolonged contacts.

Since now it was apparent that the complexes which had been intended as immunizing agents were actually infectious, we undertook to determine what numbers of bacilli could have escaped the centrifugation and filtration procedures employed in the preparation of these substances. For this purpose the total residual lyophilized complexes were dissolved in saline and ultracentrifuged at 25,000 r.p.m. for one hour. No visible sediments were thrown down, but the bottoms of the tubes were carefully scraped and smears made of the scrapings. These were examined for acid-fast bacilli. As shown in Table III, very few tubercle bacilli were encountered, although in every case the sediments represented amounts of complex greatly in excess of that received by the animals.

TABLE III.
Results of Search for Tubercle Bacilli in the Ultracentrifugal Sediments of Complexes.

Complex	Mg of complex ultracentrifuged	Result of examination of total recoverable sediment
1. Trichloracetic acid extr. of dried bacilli	200	6 bacilli
2. Trichloracetic acid extr. fresh bacilli	35	2 possible bacilli, not distinct
3. Trypsin digest extr. dried bacilli	240	4 bacilli
4. Urea extr. fresh bacilli	35	8 "

It was now evident that these guinea pigs had developed a surprising incidence and extent of infection as the result of the very small numbers of tubercle bacilli which they had received. Since we had made frequent use of this strain of organisms ($H_{37}Rv$) for infecting guinea pigs in the past, we were able to gather from previous records the relative degrees of tuberculosis established at various comparable intervals following infection with measured doses of bacteria. Guinea pigs which had received 2 ranges of infective doses by the subcutaneous route were reviewed for this purpose. The first group had been infected with 0.75 or 1.0 mg (moist weight) of bacilli, and the second had received doses of 0.05, 0.075 or 0.10 mg. A third group of pigs was available for a much more direct and striking comparison. These animals had received acetone-dried bacilli of the same batch employed for the preparation of the trichloracetic extract complex (No. 1, Table I) and the trypsin complex (No. 3, Table I). These bacilli failed to grow on glycerine egg medium during an 8-week period of observation. The organisms were administered to 13 guinea pigs in doses of 10 mg dry weight for each subcutaneous injection. A total of 40 mg was given to each animal in 4 injections during a period of 3 months.

In order to compare these 3 groups with the animals receiving complexes, the Feldman index[12] was employed to score the gross tuberculosis observed at autopsy. This index takes account of the extent of involvement of the lymph nodes draining the region of inoculation, the spleen, liver, lungs and the tracheobronchial lymph nodes.

The average indices for each group of animals are portrayed in Table IV and Fig. 1. It may be noted that the averages of organ involvement in the animals receiving complexes were uniformly greater at the various examination intervals than those observed in the guinea pigs injected with massive doses of acetone dried bacilli, and were not far below those of the animals infected with the

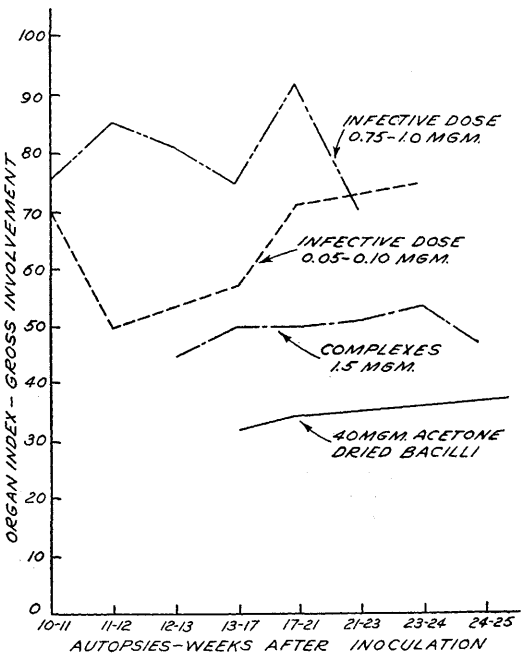

Fig. 1.

0.05 to 0.10 mg doses of fresh young culture organisms. The guinea pigs which had received 0.75 to 1.0 mg amounts of bacilli to induce infection showed more definitely advanced disease at comparable intervals following infection.

Discussion. The factors which should enter into consideration of these results are the following:

(a) The animals of the "complex groups" received a total of 1.5 mg of material of which much greater amounts revealed sparse tubercle bacilli in ultracentrifugal sediments. In contrast, the groups of guinea pigs included in the table for purposes of comparison received relatively enormous numbers of bacteria of the same strain, and in the case of the acetone dried organisms, of the same batch and at the same time.

(b) The bacilli which remained in the complexes had been subjected to chemical and physical procedures as well as aging in the lyophilized state. The organisms employed for infecting 2 of the comparative groups of animals were from 2-week-old cultures, freshly suspended in saline immediately be-

[12] Feldman, W. H., *Am. Rev. Tuberc.*, 1943, **48**, 248.

TABLE IV.
Scoring of Guinea Pig Organs for Extent of Grossly Observable Tuberculosis at Various Periods After Infection.

Wks after infection	Infected with: 0.75 or 1.0 mg fresh bacilli — No. of G pigs	0.75 or 1.0 mg fresh bacilli — Avg score	0.05-0.10 mg fresh bacilli — No. of G pigs	0.05-0.10 mg fresh bacilli — Avg score	40 mg acetone dried bacilli* — No. of G pigs	40 mg acetone dried bacilli* — Avg score	bacilli present in complexes† — No. of G pigs	bacilli present in complexes† — Avg score
10-11	23	76	2	70				
11-12	8	86	2	50				
12-13	7	81					1	45
13-17	12	74	7	58	2	32	9	50
17-21	2	92	8	71	8	34	2	50
21-23	1	70					4	51
23-24			1	75			10	53
24-25							11	47
26-32					3	38		

* Infection interval dated from 1st injection of 10.0 mg of bacilli. A total of 40 mg was eventually injected into all pigs.

† Infection interval dated from 1st injection of 0.50 mg of the various complexes. A total of 1.5 mg was eventually injected into all pigs.

fore use. The acetone-dried bacilli given to the third comparative group had been subjected to considerably less manipulation than was accorded those organisms remaining in the complexes.

(c) The infection intervals in the "complex animals" are dated from the time of first injection of these substances. The 3 injections actually spanned a period of 8 weeks, and with the small numbers of organisms involved, some of the animals may well have received no bacilli on the first, and perhaps the second, injection. The intervals after infections set forth in Table II may therefore be considerably exaggerated in some of these animals.

It appears then that the ability of the very sparse numbers of tubercle bacilli present in the complexes to produce a degree of infection greater than that found following massive doses of similarly treated organisms alone must depend upon some function of the complexes themselves. So far as can be deduced from these experiments, the complexes acted in the capacity of a virulence factor, permitting small numbers of possibly "injured" bacilli to establish themselves in the normal host and to produce progressive infection.

Whether such complexes can function in the same capacity in relation to attenuated bacilli, such as B.C.G., is being investigated at the present time. The possible role of these substances in inducing acquired resistance is also under study.

Conclusions. The Boivin type of antigenic complexes derived from the human strain ($H_{37}Rv$) of *M. tuberculosis* appear to abet generalized infection in guinea pigs receiving very small numbers of organisms. It is suggested that such complexes may function as a "virulence factor" for the tubercle bacillus in a susceptible species of host. The possible relationship of such a factor to acquired resistance to the tubercle bacillus is under investigation.