PRECIPITINS FOR THE TUBERCULIN PROTEINS OF ACID-FAST BACTERIA[1]

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INTRODUCTION

Seibert (1930) found that the tuberculin proteins of the three varieties of tubercle bacillus could be distinguished by the precipitin test. If this method could be adapted for identifying tubercle bacilli, it would be preferable to animal inoculation. The behavior of the tuberculin proteins[2] of acid-fast bacteria in the precipitin test is interesting not only as a possible means of identification of the bacilli, but also because the proteins and their derivatives are apparently the active agents in tuberculins.

METHODS

The cultures investigated included one strain of the human tubercle bacillus; one strain of the bovine; five strains of the avian, three isolated from chickens, one from a hog, and one from a cow; one strain of Johne's bacillus; and two cultures of so-called saprophytic acid-fast bacteria, one isolated from a cow and one from a hog.[3] After the growth on the synthetic medium of

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[2] By "tuberculin proteins" we mean proteins in culture filtrates as distinguished from proteins isolated directly from cells.

[3] The human strain was obtained from the Bureau of Animal Industry and has been used in our laboratory for making Old Tuberculin for twenty years. The bovine strain, isolated by Traum, was obtained from the Cornell Veterinary College. The avian strains, the Johne's bacillus, and the two saprophytic strains were all isolated in this laboratory. The Johne's bacillus grows without phlei cells. See discussion for description of saprophytic strains.

Dorset and Henley (1934) had autolyzed, usually three months after inoculation, the cells were removed by a paper and then by a Mandler filter. To the filtrate was added an equal volume of one-per-cent phenol. It was then concentrated to about one-tenth of the original volume by ultrafiltration and washed with 0.5 per cent phenol until a $BaCl_2$ test for sulphate was negative. Concentration was continued until the solution contained from 0.5 to 1.0 mgm. of protein per cubic centimeter. It was then filtered through a Berkefeld filter and handled aseptically thereafter. The amount of protein in the solution was estimated according to Seibert's (1928) method: precipitation of a 5-cc. sample with 5 cc. of 20-per-cent trichloracetic acid, centrifugation until a constant volume of precipitate was obtained, and computation for protein content from Seibert's determination on a human tubercle bacillus protein that 1 cc. of precipitate contains 0.0674 gram of protein. Nitrogen determinations on some of the protein solutions, by a modification of the micro-Kjeldahl method involving Nesslerization and colorimeter readings, showed from 10.5 to 12.5 per cent more protein than was estimated by Seibert's standardization method. As a precaution against one protein contaminating another, the Mandler and Berkefeld filters were cleaned after each use by soaking over night in a hot 0.5-per-cent sodium hydroxide solution; all glassware was cleaned with chromic acid cleaning solution, and new ultrafilters were used for each protein.

The precipitins were incited in rabbits by the subcutaneous injection of the antigens in solution, 3 to 5 cc. being given at intervals of two to three days until a total of about 25 mgm. of protein had been injected. In the first part of the work the rabbits were bled three weeks after the last injection, but later it was found that the sera taken one week after the last injection showed more antibody.

In making the precipitin tests, 0.1 cc. of the antigen solution was layered with a capillary pipette on 0.1 cc. of the antiserum. The antigen was tested in strengths starting from one gram of protein in 1250 or 2500 cc., each successive dilution being twice that of the preceding. Dilutions were usually made with 0.2

per cent salt solution, although concentrations of from 0.2 to 0.9 per cent gave no apparent differences in the results. Readings for rings were made after two hours at 37°C. and for precipitate after an additional 12 or more hours in the icebox. A lighting device described by Kanne and McCarter (1939) was used in examining for rings and precipitate.

In carrying out the precipitin absorption tests, preliminary tests were made with a constant volume of serum and varying quantities of antigen to determine the ratio necessary for the maximum amount of precipitate. In making the absorptions, apparently as much precipitate was formed if incubation was carried out wholly at 0° as if carried out partially at 37°.

RESULTS

The results of the precipitin tests with undiluted antisera and the tuberculin proteins of the various acid-fast bacteria are given in table 1. The tests with the human and bovine antisera are not conclusive, since the proteins of only one strain of each of the three varieties of tubercle bacilli were tried. However, these results agree with Seibert's (1930) in that the proteins of the three varieties of tubercle bacilli and of the two saprophytes were all distinct. The avian protein, even though it reacted in nearly as high a dilution as the human protein with the human antiserum, could be distinguished easily because it gave much less precipitate in corresponding dilutions. With the avian and Johne's antisera, the avian tubercle bacilli from the different sources and the Johne's bacillus could not be distinguished from each other, but could be distinguished from the human and the bovine types. Although the bovine protein reacted in high dilutions with the avian and Johne's antisera, in corresponding dilutions much less precipitate was obtained with the bovine protein than with any of the avian proteins or the Johne's protein. Apparently comparable amounts of precipitate with Johne's and avian proteins were obtained in both Johne's and avian antisera.

Tables 2 and 3 show attempts to differentiate the Johne's and avian proteins by precipitin absorptions and by antiserum

TABLE 1

Precipitin tests with tuberculin proteins and their antisera

ANTISERUM (UNDILUTED)	ANTIGEN TITER*									
	Human t.b.	Bovine t.b.	Avian t.b. 1 (chicken)	Avian t.b. 2 (chicken)	Avian t.b. 3 (chicken)	Avian t.b. 4 (cow)	Avian t.b. 5 (hog)	Johne's bacillus	Saprophytic acid-fast (cow)	Saprophytic acid-fast (hog)
Human	10,000		5,000						0	0
Bovine	10,000	40,000	5,000						2,500	0
Avian 1	0		20,000						0	0
Avian 2	10,000	80,000	160,000	320,000	80,000	40,000	160,000	320,000	20,000	10,000
Avian 4	0	40,000	80,000	80,000	40,000	80,000	80,000	80,000	10,000	
Johne's	10,000	80,000		160,000	160,000	80,000	160,000	160,000		5,000
Saprophyte (cow)	0	0	5,000						40,000	0

* The titer is given as the highest dilution (expressed as cubic centimeters of solution containing 1 gram of protein) of the protein solution showing a ring or precipitate as compared with a control of antiserum and salt solution. The titers for rings and precipitate were usually the same, although, when the lighting device was used, definite rings could sometimes be seen with higher dilutions than showed precipitates.

TABLE 2

Precipitin absorptions

TUBERCULIN PROTEIN WITH WHICH ANTISERUM ABSORBED	ANTIGEN TITER					
	Human t.b.	Bovine t.b.	Avian t.b. 2 (chicken)	Avian t.b. 3 (chicken)	Avian t.b. 4 (cow)	Johne's bacillus
Antiserum for the tuberculin protein of avian t.b. 4 (diluted 1 to 4)						
Salt solution control	0	640,000	320,000	320,000	640,000	640,000
Avian t.b. 4		0	0		0	0
Avian t.b. 3		0	0	0	0	0
Bovine t.b.		0	10,000	80,000	160,000	80,000
Antiserum for the tuberculin protein of the Johne's bacillus (diluted 1 to 4)						
Salt solution control	5,000	160,000	80,000	160,000	80,000	320,000
Avian t.b. 4	0	0	0	0	0	0
Avian t.b. 3	0	0	0	0	0	0
Bovine t.b.		0	20,000	80,000	80,000	160,000

TABLE 3

Precipitin tests with diluted antisera

ANTISERUM (DILUTED 1 TO 32)	ANTIGEN TITER					
	Bovine t.b.	Avian t.b. 2 (chicken)	Avian t.b. 3 (chicken)	Avian t.b. 4 (cow)	Avian t.b. 5 (hog)	Johne's bacillus
Avian 4	0	40,000	80,000	320,000	0	80,000
Johne's	0	0	40,000		160,000	160,000

dilution. In the precipitin absorption tests, absorption of both avian and Johne's antiserum with bovine protein removed only bovine-reacting precipitins, while absorption with avian protein removed bovine, avian, and Johne's precipitins. The Johne's protein never completely absorbed precipitins for either the avian or Johne's proteins and is, therefore, not included in the table. When the antisera were diluted 1 to 32, as in table 3, the bovine-protein-reacting precipitins were eliminated but the Johne's and avian were still not distinguishable. Further dilution eliminated both Johne's and avian precipitins.

DISCUSSION

That the proteins are the active antigens in tuberculins has been assumed in the interpretation of our results. This assumption is made on the basis of the finding by Seibert and Munday (1931) that the antigenicity of tuberculins in the precipitin test is correlated with high nitrogen content and large molecular size rather than with carbohydrate content, although polysaccharide is combined in some way with the protein in ultrafiltered tuberculins. This polysaccharide is removed from the protein derivatives in the P.P.D. (Purified Protein Derivative) tuberculin by repeated precipitation of the protein solution with trichloracetic acid. Since we wished to alter the protein as little as possible, and were interested in finding a test for identifying acid-fasts which would involve as few manipulations as possible, we did not remove the carbohydrate from the protein solution.

On the basis of our results, human, bovine, and avian tuberculin proteins could be distinguished from each other by the precipitin test. The separation of human from avian protein is evident from table 1, since the avian always gave much higher titers with avian and Johne's antisera. The bovine was separated from the avian on the basis of a much higher titer with bovine antiserum and much less precipitate with avian or Johne's antiserum in comparable dilutions. Also, the bovine protein did not absorb all the avian or Johne's precipitins from either the avian or the Johne's antiserum but only reduced them slightly. (We did not test the capacity of the bovine protein to absorb com-

pletely the bovine antiserum, and therefore would not draw conclusions from the absorption test alone, since the antigen in question has not been shown to absorb completely its own or a heterologous antiserum.)

The proteins of the three avian strains from chickens, the strain from the cow, and the strain from the hog, all behaved so nearly alike in the precipitin test as to be indistinguishable. Some variations do occur in the behavior of the different avian proteins in the antisera for the avian tubercle bacillus strains 2 and 4, and in the diluted antiserum for strain 4. These variations can be attributed to varying amounts of different proteins in the same tuberculin or of different reactive groups on any one protein. Seibert, Pedersen, and Tiselius (1938) have found that even the P.P.D. tuberculin contains several proteins or protein derivatives of different molecular weights. The bovine protein apparently reacts in avian antisera with the antibody for only part of the proteins or reactive groups.

The surprising result was that the Johne's bacillus protein could not be distinguished from the avian tubercle bacillus proteins in either undiluted or diluted antisera. The precipitin absorption tests gave corroborative evidence that the tuberculin proteins of the Johne's bacillus and of avian tubercle bacilli are closely similar, since the proteins of the avian strains 3 and 4 removed all precipitins from the antisera for the Johne's protein and for the avian (strain 4) protein.

The Johne's bacillus and the avian tubercle bacillus have widely different cultural characteristics and pathogenic capacities. Evidently, however, they have a common protein and therefore cannot be identified by the precipitin test. Consequently the test can not be used by itself to identify an unknown acid-fast, but can be used to give evidence supplementary to the cultural and pathogenic characteristics. Thus, the culture of avian tubercle bacilli from the cow, designated as strain 4, reacts as an avian or Johne's strain in the precipitin tests; has the cultural characteristics of the avian tubercle bacillus; is pathogenic for rabbits and not for guinea pigs; but does not produce pro-

gressive tuberculosis in chickens (a full description of this culture will be published).

The similarity of the tuberculin proteins of the Johne's bacillus and the avian tubercle bacillus is interesting because it gives a stronger basis for the use of avian tuberculin in the diagnosis of Johne's disease, which Hagan and Zeissig (1927–28) have advocated on the basis of clinical findings. It had been thought that this might be because both avian tubercle bacilli and Johne's bacilli are so often isolated from animals having Johne's disease. On the basis of the precipitin test, the common proteins probably account for the success of the diagnostic test.

That neither of the two saprophytes tested is a variety of tubercle bacillus was established by the precipitin test. The one microorganism was isolated from a no-visible-lesion tuberculin-reacting cow. Injection of this culture sensitized another cow to human tuberculin, and Feldman (1933) found that it sensitizes chickens to avian tuberculin. The other saprophyte was isolated from a hog, from lymph nodes showing caseous pinhead-sized tubercles. Neither culture is pathogenic for rabbits, chickens, or guinea pigs, and both cultures have quite similar cultural characteristics. The protein of the saprophyte from the hog does not give any cross reaction in the antiserum for the protein of the saprophyte from the cow; indicating that the two saprophytes are different species.

The occurrence of cross-reactions with the avian tuberculin protein in the antisera for the mammalian (human and bovine) tuberculin proteins, and with the mammalian proteins in the avian antisera, is in agreement with the findings that all human beings sensitive to mammalian tuberculin react to avian tuberculin (McCarter, Getz, and Stiehm (1938)), and that cattle infected with avian tubercle bacilli react to mammalian tuberculin (McCarter, Beach and Hastings (1937)).

Since the tuberculin proteins of the three varieties of tubercle bacilli, of the Johne's bacillus, and of the two saprophytes all reacted in more than one heterologous antiserum, it is doubtful whether a tuberculin test with any of the available tuberculins

can be used to diagnose for infection with a specific acid-fast microorganism; i.e., whether a reaction to a specific tuberculin necessarily means that the animal tested has been infected with the acid-fast from which the tuberculin was made.

CONCLUSIONS

1. The tuberculin proteins of the human, the bovine, and the avian tubercle bacillus are distinguishable by the precipitin test.

2. The tuberculin proteins of the avian tubercle bacilli isolated from the chicken, the cow, and the hog, and the protein of the Johne's bacillus can not be differentiated by the precipitin test.

3. The tuberculin proteins of two so-called saprophytic acid-fast bacteria, one isolated from the cow and one from the hog, are distinguishable from each other and from the proteins of the tubercle bacilli and of the Johne's bacillus.

4. The precipitin test with tuberculin proteins as antigens is useful in identifying unknown acid-fast bacteria when considered in conjunction with cultural and pathogenic characteristics. Further purification of the protein solutions seems necessary before the precipitin test can replace other methods of identification of acid-fasts.

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