THE PRECIPITIN, COMPLEMENTBINDING, AND ANTI-OPSONIC TESTS IN TUBERCULOUS AND NORMAL CATTLE.

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The recognition of latent tuberculosis, difficult as it often is in the human subject, is of necessity more difficult in the case of cattle. It is especially in early disease that the serum reactions prove often most useful as an aid to diagnosis, but in cattle a proof is also often desired that no pronounced disease is present, because it is by no means of rare occurrence that an apparently fat and healthy animal must be rejected after slaughter.

We have in tuberculosis a large number of immunity reactions, some of which are associated with the early stages of the disease, and others which are characteristic only of the end. It is well to keep this clearly in view, otherwise too much is apt to be demanded of a particular test, with consequent disappointment. It is doubtful if any single test exists which is absolutely characteristic of tuberculosis, and in all stages of the disease.

Even the tuberculin injection test, which is generally held to give the best results (Szaboky (1909, p. 274), for example, found that it gave the greatest number of positive results of 11 different serum and bacteriological tests applied by him), is yet regarded by many practical men as unreliable. The percentage of slaughtered cattle showing macroscopic tubercle varies, according to Ostertag, between 6·34 and 45 %. The percentage of cattle reacting to the tuberculin test is however infinitely higher. Siedamgrotsky tested 259 cattle and obtained a typical reaction in 76 %. Bang and Nocard in Denmark

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and France found 2/3 to 3/4 of animals examined affected. Similar results have been obtained in other countries. It is a well-known fact that animals reacting to tuberculin have been slaughtered, and no macroscopic evidence of tubercle has been found upon examination. Undoubtedly the explanation of this depends upon the delicacy of the test, so that by its means minimal traces of disease are made evident. On the other hand the tuberculin reaction may entirely fail in the later stages of the disease. Animals suffering from advanced tuberculosis, which after slaughter have been found to be riddled with the disease, have often failed to react to injections of tuberculin. The cattle-dealer who trusted to the reaction to give reliable information as to whether his meat would be saleable or not naturally lost faith in the test. But in spite of this the reaction as an indication of the presence of early or latent tuberculosis remains of the greatest value.

Many serum reactions in tuberculosis, like the tuberculin injection test, seem to have the same drawback, *i.e.* that a percentage of apparently normal sera also react. A reaction regarded by many as characteristic rather of early than of late disease, the agglutinin reaction, introduced by Arloing (1898, p. 1398), was given in the hands of that author by 22 % of normal human persons examined, and 10 out of 50 normal cattle (1901, p. 712). Beck and Rabinowitch (1901, p. 145), on the other hand, obtained the reaction in practically all slaughtered cattle examined whether tuberculous or not.

Whether the agglutinin and precipitin tests depend upon the same immune substance is still under discussion in the case of other diseases. In the case of tubercle there is a somewhat strong probability that the two are identical. The agglutination tests as described by Arloing and Koch (1901, p. 821), were carried out with serum in somewhat high concentrations, 1 : 5, 1 : 10, 1 : 20. The precipitation test as I have used it (1910, p. 88), was carried out with human serum diluted 1 : 42, and apparently at such dilutions fewer normal sera react, because of 301 normal sera only 12·3 % gave a decided precipitate, while of 381 tuberculous sera, 46·7 % reacted strongly. The greatest percentage of positive results given by these human sera were yielded by chronic cases. Advanced sera were more often negative than early sera, and nearly three times as often negative as chronic sera. The precipitin reaction in the case of human sera appears to be especially characteristic of chronic tuberculosis.

A tubercle reaction which I examined at the same time as the precipitin test in human serum was the complementbinding test for

the presence of antibody in human serum; this I found more commonly present in advanced disease, and often in cases where the precipitin test failed. A similar experience was obtained with Marmorek's test (1911) for the antigen in urine by means of complementbinding. Here again the test appeared more characteristic of advanced disease.

Another reaction, which Fornet and I (1909, p. 138) believed to be characteristic rather of advanced sera, was called by us the antiopsonic test as it depends upon the presence of an antagonism in heated serum to the action of fresh opsonin.

An antagonism between heated serum and fresh has been observed in the case of various immune reactions. This antagonism was described first by Neisser and Doering (1901, p. 595), in the case of complement in disease, and studied by v. Bergmann and Savini (1907, p. 817), Eva Hoffmann (1907, p. 704), and others. Camus and Payniez (1901, p. 730) have met with it in the case of simple isolysins apart from complement, and Welsh and Chapman (1907, p. 465) have described an antiprecipitin which as it is produced by a temperature above that required for inactivation they do not regard as merely a precipitoid. In the case of ferments Cramer and Bearn (1906, p. 36), Pollak (1905, p. 95), and Schwarz (1905, p. 524), have had similar experience.

Fornet and I (1907) met with this phenomenon in the case of opsonins and as already stated we ascribed it to the appearance of an anti-opsonin. The same result has been since then observed by various authors especially where concentrated inactive serum has been used (Rosenthal (1909), Pribram (1910, p. 1131)), but they hesitate to adopt the theory of an actual anti-opsonin. Similar results have been obtained by Hektoen and Ruediger (1905, p. 128), who found absorbed opsonin inhibitive after heating, and by Haetjens (1907, p. 560), who after absorbing bacteria with heated serum found that they were not opsonised by fresh serum, which was perfectly able to opsonise untreated bacteria.

Fornet and I were disposed to lay some stress upon the importance of this phenomenon on account of the fact that this antagonistic substance was produced not only by heat but also in the neighbourhood of bacteria, and even of artificial membranes. The inactivation of opsonin in the presence of reed membranes has recently been confirmed by Pribram (1910, p. 1131), while Ledingham (1907, p. 482) has noticed the appearance of an antagonistic influence in serum through which bacteria had been passed, which he ascribed to bacterial products. The inactivation of ferments in contact with artificial membranes has been

noticed by Slosse and Limbosch (1909, p. 417), and myself (1910, p. 301), a process which was not explained by simple absorption, and which was accompanied by the appearance of antiferments. The production of antiferments by natural membranes with a view to their own protection has been described by Weinland (1902, p. 45), in the case of the stomach and intestines, while Kantorowicz (1909, p. 897) has described an inactivation of trypsin in the membranes of bacteria. Again, Pfeiffer and Friedberger (1905, p. 1145), and Bail and Kikuchi (1905, p. 275), have noticed an analogous behaviour on the part of bacteria against amboceptor, and Weil (1905, p. 291) has described the same in the case of agglutinin. On account of the fact that artificial membranes can powerfully exert this influence at least upon complement, opsonin, and certain ferments, the effect depending greatly upon the physical condition of the membranes, transparency etc., the action of such membranes as a whole may be taken as due in part to a physical property of membranes upon absorbed substances. The possibility of the inactivation of immune substances and the actual production of antagonistic agents by bacterial membranes in the human body must not be lost sight of, as it seems to present a weakness in the defences of the organism.

It appears highly probable that the absorption of opsonin by bacteria in the absence of leucocytes is unnatural, and if occurring in the animal body, unfortunate. A large number of authors at the present time, for example Neufeld (1904, p. 1458), and his collaborators, Hektoen (1909, p. 66), Rosenow (1906, p. 683), Fornet and myself (1909, p. 156), regard the action of phagocytic agents in the serum as an agglutination of bacteria to leucocytes. This view is held partly because of the great rapidity with which phagocytosis takes place, but mainly because the clumping of masses of leucocytes and bacteria together, which is an invariable accompaniment of phagocytosis where the process is not prevented by the presence of large numbers of red cells, has also been observed with dead leucocytes.

Hektoen (1906, p. 19), Weil and Tsuda (1907, p. 1038), Levy and Fornet (1906, p. 1039), and Fornet and myself (1909, p. 166), have noticed that culture filtrates hinder phagocytosis by acting against the leucocytes.

It appears likely that while the bacterial products keep the leucocytes at bay and counteract agglutination an unnatural absorption of opsonic substances is taking place followed by an inactivation of opsonin and production of antiopsonin in the bacterial membrane. The partial

disappearance of opsonin in the bacterial wall, after absorption in vitro, has been pointed out by various authors, Dean (1905, p. 506), Meyer (1908, p. 951), Centanni (1908, p. 140), Sellards (1908, p. 308), Hektoen (1909, p. 74), and Fornet and myself (1909, p. 147).

The antiopsonin is most easily and rapidly produced by heat and is therefore best measured in heated serum.

In the following experiments I have examined the sera of 408 cattle (including 30 tuberculous) for the precipitin reaction, both with bovine and human tubercle extract, of 80 cows (including 18 tuberculous) with the complementbinding test for antibody, and 118 cows (including 20 tuberculous) with the antiopsonic test, with a view to testing their value as diagnostics of the presence of tuberculous disease in cattle, and incidentally to note the number of normal animals reacting.

The precipitin test.

The following results were obtained with the precipitin test, the expression normal being used to denote animals in which no macroscopic tubercle was found after slaughter.

Method. The method employed was similar to that which I have already described in a former paper (1910, p. 88). Bovine and human tubercle bacilli were ground with distilled water and the mixture shaken and kept at 37° for 36 hours, sufficient NaCl and phenol being added to render the concentration of NaCl 0·85 %, and of phenol 0·5 %. The extract was then filtered through a Berkfeld filter. One drop of serum was placed in a narrow tube and diluted with 20 drops of physiological salt solution to 1/21. Seven drops of diluted serum were removed into a second tube, and seven into a third. This dilution of the serum to 1/21 was not critical in any way. The dilution was chosen simply because while not too weak it yet gave a convenient amount of fluid in the tube (which was of the narrowest size) for a precipitate to be conveniently examined.

An equal quantity of bovine tubercle extract was added to the first tube, of human tubercle extract to the second, while the third (control) tube received a solution containing 0·85 % NaCl and 0·5 % phenol alone. Every tube therefore contained phenol. As it was found that bovine serum is less liable to precipitate with phenol alone than is the case with human serum, controls containing tubercle extract without phenol were not used, only controls containing phenol without tubercle extract being employed.

The tubes which had been filled with sterile precautions were stoppered with sterile wads and placed at 37° for 12 hours, after which they were examined for precipitates.

Normal Cows' Serum.

No. of animals tested	Tested with	Result				Percentage	
		Negative	Doubtful	Positive	Strong	Positive	Negative
180	Bovine extract in 0·85 % NaCl and 0·5 % phenol	119	9	30	22	29	66·1
180	Human extract in NaCl, etc.	134	7	24	15	21·6	74·4
180	0·85 % NaCl and 0·5 % phenol alone	172	5	3	—	1·66	95·5

Fourteen of these 180 sera reacted to bovine tubercle extract alone not to human, and 4 reacted to human tubercle extract but not to bovine.

Normal Bullocks' Serum.

No. of animals tested	Tested with	Result				Percentage	
		Negative	Doubtful	Positive	Strong	Positive	Negative
198	Bovine extract in 0·85 % NaCl and 0·5 % phenol	151	14	29	4	16·66	76·2
198	Human extract in 0·85 % NaCl and 0·5 % phenol	164	11	21	2	11·6	82·82
198	0·85 % NaCl and 0·5 % phenol	184	11	2	1	1·5	92·98

Ten of these 198 sera reacted alone to the bovine tubercle extract, one very strongly, without showing any trace of precipitate with human tubercle extract. Two reacted alone to human extract without responding to bovine.

From the above results it will be noticed that,

(1) a somewhat large percentage of apparently normal animals reacted,

(2) a larger number of cows reacted than of bullocks,

(3) that while the majority reacting did so to both human and bovine tubercle extract, a certain number reacted only to one, generally the bovine extract. (It may be mentioned that precipitates to the bovine extract were generally stronger than to the human.)

(4) the precipitates to 0·5 % phenol in physiological salt solution were extremely few in number. (In this respect the serum of cattle differs greatly from human serum, which precipitates almost as frequently

with phenol as with tubercle extract which contains no phenol, both precipitates occurring in the same serum.) The reaction is correspondingly more reliable.

I should like to mention that Mr Thomson, the Veterinary Inspector who was so kind as to collect the sera and examine the carcases, gave the specimens to me in batches without special information about them. He was often able to notice in the results afterwards that reacting animals had come from the same source, where they must have been exposed to the same conditions. On this account and also because of the fact that more cows react, which are well known to be more exposed to infection, it is very probable that we have here to deal with cases of early tuberculosis so extremely slight as to be unrecognisable at the ordinary examination after slaughter, rather than with an inaccuracy of the method.

Tuberculous cattle.

The only tuberculous sera which I was able to obtain were extremely small in number, only 30. The majority of these were from advanced cases.

No. of animals tested	Tested with	Result				Percentage	
		Negative	Doubtful	Positive	Strong	Positive	Negative
30	Bovine extract in 0·85 % NaCl and 0·5 % phenol	8	6	10	6	53·3	26·6
30	Human extract in 0·85 % NaCl and 0·5 % phenol	16	4	7	3	33·3	53·3
30	0·85 % NaCl and 0·5 % phenol	28	2	—	—	Nil	93·3

One case acted very strongly to human tubercle extract but not at all to bovine, and one which acted to both did so more strongly to human than bovine. Eight were positive to bovine extract but not human. The bovine precipitates were usually the stronger, but the results obtained were on the whole somewhat weak, and the percentage of positive results fewer than might have been expected. This was probably due to the fact that the sera were almost all from cases of advanced disease.

One case might be described as of interest. A cow was found to be spitting up a quantity of sputum in which tubercle bacilli were discovered. The animal was condemned and slaughtered. After slaughter no evidence of tubercle could at first be detected, the organs appearing healthy. Congestion of the trachea was then observed and tubercle

bacilli were found microscopically in the mucous membrane. The serum of this animal precipitated very strongly indeed to bovine tubercle extract, but not at all to human tubercle extract. This was the only case of early tuberculosis examined.

The complementbinding test.

Method. After the tubes prepared as above described for the precipitin reaction had been examined and any precipitates noted, complement was added in an amount sufficient to haemolyse 1·0 c.c. of 4 % sheep's corpuscles, *i.e.* 0·1 c.c. of guinea-pig serum. The tubes were then placed in the incubator again for an hour and at the end of this time 0·5 c.c. of amboceptor (immune rabbit's serum) and 1·0 c.c. of 4 % sheep's corpuscles added. The mixture was again placed at 37° for two hours.

Eighty sera were tested in this way, including 18 tuberculous.

Normal Cows' Serum.

No. of animals	Tested with	Result:—Haemolysis			Percentage	
		Complete	Partial	Absent or trace	Binding	Not binding
62	Bovine extract in 0·85 % NaCl and 0·5 % phenol	50	9	3	18·35	80·64
62	Human extract in 0·85 % NaCl and 0·5 % phenol	50	10	2	19·35	80·64
45	0·85 % NaCl and 0·5 % phenol	45	—	—	—	100

Eight normal sera which did not precipitate with human tubercle extract (12·9 %), bound complement in its presence (of these sera 5 had however precipitated to bovine extract). On the other hand 3 sera which had precipitated with bovine and human tubercle extract failed to bind complement.

Tuberculous Cows' Serum.

No. of animals	Tested with	Result:—Haemolysis			Percentage	
		Complete	Partial	Absent or trace	Binding	Not binding
18	Bovine extract in 0·85 % NaCl and 0·5 % phenol	2	2	14	88·88	11·11
18	Human extract in 0·85 % NaCl and 0·5 % phenol	5	4	9	72·22	27·77
18	0·85 % NaCl and 0·5 % phenol	18	—	—	—	100

Ten tuberculous sera (55 %) which had not precipitated with human tubercle extract, bound complement in its presence (of these sera 3 had however precipitated with bovine extract).

The antiopsonic test.

Method. Wright's method was employed. The leucocytes were from human blood, the bacillary emulsion from Allen and Hanbury's moist preparation.

The middle point between two estimations of the same normal bovine serum, measured at the same time, was taken as 1. A group of sera under investigation were heated for half an hour at 58° to 60°, and were then tested as to their power of reducing the opsonic value of this fresh normal serum.

It was found that while with 1 part of fresh normal serum, 1 part of physiological salt solution, 1 part of leucocytic and 1 part of bacillary emulsion the index stood at 1, when the physiological salt solution was replaced by heated bovine serum the resulting opsonic value varied between 0·1 and 1·27, but was generally lower than the control 1. By using 1 as a standard it was made possible to compare all these different estimations made on different days with different sera.

Altogether 118 heated cows' sera were tested including 20 tuberculous.

Normal Cows' Serum.

Number tested 98.

Index changed from 1·0 to between	No. of sera	Percentage	
0·1 and 0·2	1		
0·2 ,, 0·3	3	18·3	
0·3 ,, 0·4	8		
0·4 ,, 0·5	6		75·5
0·5 ,, 0·6	5		
0·6 ,, 0·7	14		
0·7 ,, 0·8	17		
0·8 ,, 1·0	10		
1·0 ,, 1·1	17		
1·1 ,, 1·2	6	24·4	
1·2 ,, 1·3	1		

In 75·5 % of cases the opsonic value of normal bovine serum was lowered by the heated serum. In 18·3 % of cases it was reduced to below one-half its former value.

Of these 18 sera which were so powerfully antagonistic 3 reacted slightly to the precipitin test, none bound complement. As a negative precipitin test points either to advanced disease or to absence from disease, and in these cases certainly no marked disease was present, the extreme antagonistic power of these sera indicates rather an inborn susceptibility, a flaw in the protective mechanism rather than necessarily a proof of disease.

Tuberculous Cows' Serum.

Number tested 20.

Index changed from 1·0 to between	No. of sera	Percentage	
0·1 and 0·2	2		
0·2 ,, 0·3	5	65	
0·3 ,, 0·4	3		
0·4 ,, 0·5	3		85
0·5 ,, 0·6	1		
0·8 ,, 0·9	1		
0·9 ,, 1·0	2		
1·0 ,, 1·1	2	15	
1·1 ,, 1·2	1		

In 85 % of cases the opsonic value of fresh serum was lowered, in 65 % of cases to below one-half its former value. The difference between normal and tuberculous serum is striking. All the serum which did not lower the opsonic value, precipitated and bound complement, while of the 13 which lowered the value by more than a half, as many as 9 did not precipitate.

Conclusions.

Advanced tuberculous bovine serum, like human, does not respond very well to the precipitin test. On the other hand a certain percentage of animals without any macroscopic evidence of tubercle, do react. On this account the reaction, although undoubtedly valuable as a prophylactic, does not afford very reliable information as to the condition of an animal about to be slaughtered for meat. Combined with the complementbinding test which appears to be more characteristic of advanced than of early disease (the precipitin reaction belonging rather to the early and intermediate stages), it should prove of considerable value. It is interesting that bovine tuberculous serum does not precipitate with 0·5 % phenol, in the same way as human tuberculous serum.

The antiopsonic reaction can hardly be said to be characteristic of tuberculous disease. It represents apparently a flaw in the normal protective mechanism, and if especially present in advanced tuberculous sera is probably only so because the individuals possessing this pre-antiopsonin were rendered thereby more susceptible to the progress of the disease.

If the precipitin reaction is on the whole perhaps prognostically more favourable, the antiopsonic reaction is probably prognostically unfavourable.

I should like to express my thanks to Mr Thomson, Veterinary Inspector, Edinburgh Slaughterhouse, for his kindness and the great trouble he has taken in collecting the specimens of blood, and examining the carcases.

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