IMMUNOLOGICAL PROBLEMS IN PARATUBERCULOSIS

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Paratuberculosis, or Johne's disease, is caused by an acid-fast bacillus, *Mycobacterium paratuberculosis*, and affects several species of ruminants, particularly sheep and cattle. It consists of an insidious and chronic inflammation of the intestinal mucosa and the mesenteric lymph glands draining the affected part. The inflammation may lead to extreme thickening of the mucosa and submucosa. It is most commonly located in the lowest part of the ileum adjacent to the ileocecal junction, but in advanced cases the lesions may extend into the cecum. The causative organisms may usually be found in the diseased tissue, often in enormous numbers. Even advanced lesions of paratuberculosis are sometimes found, however, in which only few or no acid-fast bacilli can be demonstrated microscopically.

From six months to several years usually elapse after effective transmission of paratuberculosis until symptoms of the disease become clinically noticeable, and the course of the clinical phase of disease is also very protracted. When the disease reaches a stage where clinical symptoms are in evidence, it is fatal in the large majority of cases. On the other hand, there is reason to believe that latent infections which never become clinically recognizable are quite common in infected herds, or in other words that many of the animals, that are infected develop immunity early and never show clinical signs of the infection. Taylor, in England (1), isolated *M. paratuberculosis* from the ileocecal lymph nodes of 15 per cent of healthy cattle that came to the slaughterhouse. He believes that the present comparatively ineffectual methods for isolating the organism do not detect nearly all infected animals and that many more "healthy" animals infected areas may be expected to be infected, perhaps 25 to 30 per cent. Rankin (2) also found a considerable number of cattle latently infected.

Epizootiological evidence is also available to suggest that latent infections are common. For example, 20 sheep were introduced into Iceland from Germany in 1933. They came from a herd that was not known to be infected with paratuberculosis, and the imported sheep were clinically healthy. Five of these twenty sheep transmitted the disease to healthy contacts and were thus proven to be carriers of the infection (3, 4). Paratuberculosis had not been known to occur in Iceland previously.

Paratuberculosis is of great economic consequence to sheep and cattle raising in many countries in all parts of the world, and methods of control have therefore been actively sought. With few exceptions, which will be mentioned later, the only method employed to control the spread of the disease has been attempts at early diagnosis and subsequent destruction of infected animals. It appears that this method has nowhere proved very effective. The reason for this is, in the writer's opinion, that early diagnosis of clinical cases is difficult and also that latent infections are common, and healthy carriers therefore play an important role in the epizootiology of paratuberculosis.

Most immunological studies of paratuberculosis have either been aimed at developing diagnostic procedures or they have attempted to lay the foundations of a satisfactory procedure for active immunization. This is natural, as measures taken to control the spread of the disease must necessarily be based on immunological methods of diagnosis or prevention. If a satisfactory method of active immunization could be developed, this would probably be the method of choice.

It is natural that immunological studies of paratuberculosis should have been fashioned after corresponding work on infections with other species of mycobacteria, such as mammalian tuberculosis, avian tuberculosis and leprosy. That is, workers have tried to develop satisfactory methods for testing skin sensitivity in paratuberculosis with tuberculin-like products, to develop a complement fixation reaction for diagnostic purposes, and finally to develop a vaccine capable of provoking active resistance to paratuberculosis.

A general description of the various aspects of
paratuberculosis as it appears in cattle and sheep will not be given here. Such information may be had in a number of textbooks (5, 6, 7). The subject matter of the present review will be discussed in three sections: (a) allergy to tuberculin-like substances, (b) complement fixation tests as a diagnostic tool, and (c) attempts to provoke active immunity.

1. ALLERGY TO TUBERCULIN-LIKE SUBSTANCES

Animals infected with paratuberculosis will at a certain stage of the disease be sensitive to the injection of suitable mycobacterial products. Oluf Bang discovered in 1909 (8), i.e., before the organism of paratuberculosis was artificially cultivated, that tuberculin made from Mycobacterium avium will elicit an allergic reaction in animals with paratuberculosis.

Shortly after the successful cultivation of M. paratuberculosis by Twort in 1911 (9) attempts were made to prepare paratuberculins or “johnines” from such cultures. The early attempts apparently were not very successful, but as it gradually became possible to get luxuriant growth of M. paratuberculosis on the surface of entirely synthetic liquid media (11, 12), the possibility arose of preparing more potent “paratuberculins” for use in the allergic test on infected animals. In later years several workers have prepared “purified protein derivatives” from paratuberculins, using similar techniques, as is now common practice with M. tuberculosis var. hominis and var. bovis, and have found that these are also useful for the allergic test (13, 14, 3, 15).

The sensitivity of paratuberculous animals to avian tuberculin may be demonstrated by injecting them with comparatively large doses of avian tuberculin and measuring the body temperature at certain intervals for 8 to 24 hours afterwards. Bang injected subcutaneously 10 ml of a 20 per cent solution of a heat-concentrated preparation of avian tuberculin similar to the human OT (8). The shape of the temperature curve after the inoculation in cattle may be seen in figure 1 which is based on tests in 124 animals (16). The avian tuberculin may be injected intravenously (17). A dose of 10 ml concentrated tuberculin has been used. The graph showing the rise in temperature after the intravenous injections (figure 2) is based on data from Hagan’s paper (17).

It seems that after subcutaneous injection the average curve (figure 1) reaches its maximum from 11 to 15 hours after the injection. After intravenous injection the average curve (figure 2) begins to rise after 1 hour, reaches a peak after 5 to 6 hours, and then falls gradually. It is not clear whether this difference in the interval after the injection until the fever curve reaches its peak is due to the different routes of inoculation, or to the difference in dose of tuberculin employed, or possibly both. A direct comparison of the intravenous and subcutaneous routes, employing a standardized quantity of a purified product, would be needed to elucidate that point.

A drawback of using the rise in body temperature to indicate an allergic reaction is that it can not be exactly predicted how soon after injection the temperature of any given animal will reach its peak, and this varies greatly. The period of high temperature in each animal is commonly only 2 to 6 hours or less, so that it is necessary to take the temperature of the animals several times in order not to miss a small but significant rise. With subcutaneous injection it seems from Bang’s data that the peak may be reached as
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1956

3

Temperature

0°C

41

40

39

38

0 5 10

Hours after injection

Figure 2. Curve showing the average rise in body temperature in cattle after intravenous injection of avian tuberculin. After Hagan and Zeissig (17).

early as 9 hours or perhaps earlier and as late as 20 hours after the injection. After intravenous injection, the temperature seems to rise more quickly and to last for a shorter period than after the subcutaneous injection, but then this question would need a more direct comparative test.

Beach and Hastings measured the temperature every hour from 1 to 10 hours after the intravenous injection of paratuberculins (18). In tests on 51 positive animals the hours of maximum temperature in the individual animals were distributed as shown in figure 3, which is based on data given by these workers (18). From these and other data it seems that it would be desirable to take the temperature hourly from the third to the tenth hour after the intravenous injection of paratuberculin.

A rise of about 1°C above the pre-injection temperature has been considered significant (11). The shape of the temperature curve and the general appearance of the animal after the injection should also be considered in doubtful cases, when the outcome of the test is being evaluated. To the reviewer's knowledge, purified preparations of paratuberculin have not been used in the intravenous test.

The sensitivity to avian tuberculin may also be demonstrated by injecting a small amount (0.1 or 0.2 ml of the heat-concentrated product) intracutaneously and observing the site of injection again 48 hours later. A positive reaction is indicated by local edema and hyperemia and even by a small necrotic spot at the site of the injection. A variant of the intracutaneous test is to inject 0.1 or 0.2 ml of avian tuberculin or paratuberculin twice into the same site with 24-hour or 48-hour intervals and then to read the reaction 24 hours after the second injection (10).

In such tests it is customary to measure the thickness of a skin fold at the proposed site before the injection is made and again at the time the test is read. The difference between the two measurements indicates the increase in thick-
ness caused by the injection. Although this is undoubtedly very useful, other signs, such as redness, tenderness, and the diameter of the lesion, should also be noted, and all taken together should form the basis of judgement.

Since the beginning of bacteriological work on paratuberculosis, a number of workers have tried to produce a paratuberculin or johnin from cultures of *M. paratuberculosis* in the belief that a more specific allergic test could be developed with such material than had been possible with avian tuberculins. For a long time it proved difficult to get sufficiently luxuriant growth on fluid media so that reasonably active preparations could be prepared from such cultures. In later years, however, since it has become possible to obtain excellent growth of *M. paratuberculosis* on the surface of entirely synthetic liquid media (11, 12), several bacteriologists have prepared and tested such paratuberculins or johnins (e.g., see 11, 12, 17, 14, 19). These have been employed in the same way as already described for avian tuberculin, injected either into the skin or intravenously. In the last 10 years or so, purified protein derivatives (PPD) from cultures of *M. paratuberculosis* have been prepared, and these seem to have been tested mainly in the skin test (13, 14, 15, 20, 3).

At the present time both paratuberculins and avian tuberculins are used in field work for detecting cattle and sheep infected with *M. paratuberculosis*. It seems, of course, more logical to employ for the diagnosis of paratuberculosis a tuberculin prepared from *M. paratuberculosis*, the organism causing the disease, rather than a preparation prepared from cultures of *M. avium*, which has nothing to do with paratuberculosis. The reason why avian tuberculin has been and still is widely used for detecting paratuberculosis is probably that *M. paratuberculosis* is more difficult to cultivate, particularly on fluid medium, than is *M. avium*, and that consequently the production of paratuberculin of a uniform potency has been a more difficult task. Although some workers prefer one type of product over the other for allergic tests for paratuberculosis, little controlled evidence has been published so far to prove that one product is generally more reliable than the other, and as a matter of fact, some comparative studies of the specificity of paratuberculin and avian tuberculin indicate that they are very closely related indeed, as will be mentioned later in this review.

As to the choice between the various methods of performing the allergic tests on paratuberculous animals with either paratuberculin or avian tuberculin, it may be stated that a direct comparison of the same or identical products on a considerable number of naturally infected animals would be needed before it could be definitely decided whether the subcutaneous or intravenous injection of the tuberculin with a subsequent recording of the body temperature on the one hand, or the local intradermal test on the other hand, is a more reliable diagnostic method. At the present time it appears that the various workers choose one or the other method mainly because of the relative ease of application under the circumstances prevailing in the different localities. It seems, however, that for diagnostic work in sheep the intradermal test is used almost exclusively.

This rather unsettled state of affairs is obviously due to the considerable difficulties involved in making adequate comparative studies of diagnostic procedures of this type. These difficulties are manifold and concern both the standardization of the tuberculin-like products to be tested and the subsequent anatomical or bacteriological diagnosis of the disease in the animals used for the comparative tests. It may be stated generally that diagnostic work employing impure tuberculin preparations which have not been adequately standardized beforehand will not contribute greatly to the solution of this general question although each and all of them may yield results of practical diagnostic value. When a product or a procedure of this type is being tested, it is very important that infected or suspected animals which have been employed in comparative tests should be carefully autopsied as soon as possible after the tests have been finished, in order to compare the incidence and extent of specific lesions with the outcome of the various tests.

Earlier workers disagreed as to whether or not paratuberculous animals are sensitive to human or bovine tuberculins. This question is a part of the wider problem of cross reaction between the various types of mycobacteria. It seems now that animals sensitized to any type of mycobacteria will react to “tuberculins” prepared from any other type of mycobacteria but that much more
is needed of a heterologous product than of a homologous one to elicit an allergic reaction of standard strength. This indicates that studies of the specificity interrelationships of tuberculin-like products from various types of mycobacteria are not of much value unless such work is rather strictly quantitative.

Important studies of the specificity interrelationships of tuberculins prepared from various mycobacteria have been carried out by Green and co-workers in England (15, 20). Since 1939 these investigators have employed a simplified chemical method for preparing purified tuberculins. For example producing PPD from *M. tuberculosis* var. *hominis* involves an initial precipitation of the active principle with trichloroacetic acid and subsequent washing of the sedimented material with buffered water. The precipitated tuberculin may then be subsequently dried in acetone and ether. The dried, concentrated and purified product contains about 90 per cent protein, the remaining 10 per cent being mainly carbohydrate and nucleic acid. According to these workers the specific activity of these preparations per weight unit is so constant that the kjeldahl test on the solution of the redissolved protein is really a better comparative measure of the activity of a new preparation than direct activity measurements of the product on sensitized guinea pigs. The reason for this is that the accuracy of the guinea pig test employed for the bioassay of tuberculin is, according to Green, ±40 per cent, but the accuracy of the nitrogen determination is about ±1 per cent so that if activity per mg protein is constant as maintained by Green, the advantage of the chemical test is obvious.

Green and co-workers have also prepared by their relatively simple method PPD "tuberculins" from several species of mycobacteria, including *M. paratuberculosis*, and tested each product extensively on large numbers of guinea pigs sensitized to all the various species of mycobacteria, in order to get quantitative information on the cross-reactions between the various bacterial species (15). Table 1, which is taken from reference 20, shows the outcome of these tests.

The amount of PPD needed to elicit an allergic reaction of standard strength in a group of animals sensitized to the homologous mycobacterium is called one "weight unit" of that product and indicated by the numeral 1 in the homologous tests in the table. The other figures in the table, showing the outcome of the various "heterologous" tests, give the "specificity factors" of the various preparations. The "specificity factor" is defined as the number of units of the heterologous protein derivative required to elicit the same intradermal reaction as one unit of the homologous protein derivative.

In the table a value of 1 for a "heterologous" test (e.g., "hominis" tuberculin tested in animals sensitized to *bovis* organisms) indicates that the same amount of tuberculin which will elicit a reaction of standard strength in "homologous" animals will also elicit a reaction of standard strength in the "heterologous" animals. In such a case the heterologous product is assumed to be immunologically identical with the homologous product. The higher the figures in the table the farther removed from the homologous tuberculin is the specificity of the preparation being tested.

The table shows quite clearly that the tuberculins from *hominis, bovis* and *BCG* are closely related. Correspondingly *avtis* and *paratuberculosis* are very closely related, but *M. phlei* stands alone. In spite of these groupings there are cross reactions between all groups if a sufficient quantity of "tuberculin" is administered.

In this work the guinea pigs were sensitized by injecting into the muscle 0.5 mg living organisms of *M. tuberculosis* var. *hominis, M. tuberculosis*.

### TABLE 1

<table>
<thead>
<tr>
<th>Type of sensitisation</th>
<th>Type of PPD Test Material</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Hominis</td>
</tr>
<tr>
<td><em>Mycobacterium tuberculosis</em> var. <em>hominis</em></td>
<td>1 2</td>
</tr>
<tr>
<td><em>BCG</em></td>
<td>1 2</td>
</tr>
<tr>
<td><em>M. tuberculosis</em> var. <em>bovis</em></td>
<td>1 2</td>
</tr>
<tr>
<td><em>M. paratuberculosis</em></td>
<td>10 10 10 10 10 10 10 10</td>
</tr>
<tr>
<td><em>M. avium</em></td>
<td>20 40 40 40 40 40 40 40</td>
</tr>
<tr>
<td><em>M. phlei</em></td>
<td>150 150 150 150 150 150 150 150</td>
</tr>
</tbody>
</table>
war. *bovis*, *M. avium* and BCG, as these are pathogenic for guinea pigs. Sensitization to *M. phlei* and *M. paratuberculosis*, which are not pathogenic for guinea pigs, was achieved by injecting the killed organisms suspended in oil.

The old controversy about the presence or absence of sensitization to "human" or "bovine" tuberculins in paratuberculous animals is thus explained: heterologous tuberculins will not elicit a reaction if administered in the quantities adequate for the homologous test but will do so if a sufficient, much larger quantity is injected.

One word of caution, however, is needed here: the data on which Table 1 is based were, as already stated, obtained in artificially sensitized laboratory animals. Some cases were sensitized by infecting them with living virulent bacilli. With the bacterial species apathogenic for guinea pigs, however (*M. paratuberculosis* and *M. phlei*), sensitization was achieved by injecting the guinea pigs with killed bacilli suspended in mineral oil. As pointed out by Dr. Green himself, it is entirely possible that the sensitization obtained by injecting killed apathogenic bacteria is to some extent qualitatively different from the sensitization of a natural host going through a mycobacterial infection. The specificity interrelationship between *M. paratuberculosis* and the rest of the group may have become somewhat distorted by such irregularities, but the method employed seems to be the only one available at the present time. Hence the very great value of these comparative tests.

It would be of great practical interest if the specificity of the "tuberculins" could be increased, or, in other words, if their tendency to give cross reactions in infections with "heterologous" mycobacteria could be decreased.

Attempts have been made to increase the specificity of paratuberculins by chemical fractionation; Jones et al. (21) report that a fraction representing only 2.5 per cent of the total nitrogen of the starting material was much more specific and more potent on a weight basis than the standard product. This recent work has not yet been confirmed.

Green (15) has suggested that the different chemical groups responsible for the specific activity of the various tuberculins may be selectively attacked by chemical means and that it should thus be possible to increase or decrease the specificity of a given preparation without necessarily at the same time affecting its potency in an organism sensitized to the homologous mycobacterium. For example, Green reports (15) that McGirr has found that treatment of human or bovine PPD with chloramine-T or with ninhydrin damages the nonspecific groupings more than the specific groupings and thus renders the product more specific for sensitization to *M. tuberculosis* var. *hominis* and var. *bovis*. PPD prepared from *M. tuberculosis* var. *avium* or *M. paratuberculosis*, on the other hand, is rendered less specific by a corresponding treatment, i.e., in both instances the groups specific for *M. avium* and *M. paratuberculosis* were "inactivated" by treatment with chloramine-T and ninhydrin to a greater extent than were the groups specific for *M. tuberculosis* var. *hominis* and *bovis*. To the reviewer's knowledge the details of this work have not been published, but here is a possibility of great interest.

Measurements of the activity of paratuberculins preparation have been somewhat difficult. The skin of naturally infected, allergic cattle may be used for such titrations but their skin sensitivity is usually of a low order and subject to fluctuation. Attempts have been made to produce skin sensitivity in cattle by subcutaneous injection of living *M. paratuberculosis* in gum acacia and mineral oil (22). The animals became moderately allergic, but their sensitivity was relatively fleeting.

Glover (23) reported that guinea pigs may be sensitized by inoculating them intraperitoneally with 30 mg of a wet culture of *M. paratuberculosis* suspended in sterile mineral oil. Skin sensitivity was generally at its peak from the sixth to the tenth week after the injection and during that period it proved to be very satisfactory. Guinea pigs weighing less than 400 g were found to be unsuitable for such work, as young animals did not develop good allergy. The tests should be read after 48 hours.

Preparations of paratuberculin evaporated to 1/40 of their original volume gave readable reactions in dilutions varying from 1:2000 up to 1:8000. The accuracy of titrations carried out by this method was satisfactory.

Konst and Watson (14) reported similar results with the intraperitoneal sensitization of guinea pigs. In their experience, however, many animals perished after the intraperitoneal injection of the oily suspension. They therefore preferred injecting calcium phosphate intraperitoneally 48 hours before injecting 15 mg of bacteria by the
same route. This method proved less dangerous to the animals and in their experiments produced equally good sensitization as the oily suspension of bacteria.

Johnson and Cox (24) did not succeed in eliciting a satisfactory sensitization in young guinea pigs (250 to 350 g) by injecting them intraperitoneally with 50 mg of *M. paratuberculosis* suspended in mineral oil, thus confirming Glover's results. These authors developed a method using the chicken, which they believed developed a more reliable sensitivity than did the young guinea pigs or rabbits that they also tested. Four- to eight-week-old white Leghorn cockerels were inoculated intraperitoneally with 50 mg of wet *M. paratuberculosis* suspended in 0.5 ml of mineral oil. Six to eight weeks later the chickens are ready for the intradermal test. The injection was done into the skin of the wattle.

It is the reviewer's opinion that allergic tests have been somewhat disappointing as tools of diagnosis in natural infections with *M. paratuberculosis*. Infected animals sometimes do not become positive until the disease is rather advanced, they become negative again in the later stages of the infection, and, at least in certain animal populations, false positive reactors are common. The allergic tests are undoubtedly very useful under certain circumstances, but too much reliance should not be placed in them.

II. COMPLEMENT FIXATION TESTS AS A DIAGNOSTIC TOOL

A number of authors have studied the possibility of employing a complement fixation test for the diagnosis of paratuberculosis. Twort (25) employed a suspension of artificially cultivated *Mycobacterium paratuberculosis* as antigen and found that cattle affected with paratuberculosis would react in the test. Twort only tested a limited number of animals and came to the conclusion that such a test would hardly be of much value for diagnostic work.

Using a complement fixation reaction, Bang (26) tested cattle sera from animals in herds that were known to be free of tuberculosis but affected with paratuberculosis. As antigen in the test he employed a suspension of mycobacteria cultivated on artificial media. Comparing suspensions of *M. paratuberculosis*, *M. avium* and *M. tuberculosis* var. *hominis* (isolated from a case of spontaneous tuberculosis in a parrot), he concluded that the last named was best suited for the test. It seems that this conclusion is based on the fact that the largest number of positive reactions were obtained with this type of antigen.

Bang tested 800 sera. Less than 50 per cent of sera from animals, which had proved to be allergic to avian tuberculin, reacted in the complement fixation test. He found that 10 to 12 per cent of animals from supposedly healthy herds also reacted. His explanation for this was that many of these animals were probably latently infected. Bang stresses that the complement fixation reaction employing such antigen does not discern between infection with *M. tuberculosis* and *M. paratuberculosis*. In spite of this, his general conclusion is, "that the complement fixation test is of no small importance to the diagnosis of paratuberculosis."

Hagan and Zeissig (27) carried out a considerable number of complement fixation tests on naturally infected cattle employing a variety of antigens, among them a partially defatted tubercle bacillus antigen and an antigen prepared by the method of Witebsky, Klingenstein and Kuhn (28). For the latter, bacteria grown on artificial media are sequentially extracted with alcohol, pyridine and acetone; the residue from the acetone extraction is extracted with benzol. The benzol-soluble fraction is suspended in water, lecithin is added, and the resulting suspension is used in the test. Hagan and Zeissig, comparing some of these antigens in their serological work on cattle, found, as did the earlier authors, that the complement fixation they obtained was not specific for Johne's disease, but rather was group-specific in that it also yielded positive reactions in animals infected with *M. tuberculosis* var. *hominis*, *M. tuberculosis* var. *bovis* and *M. avium*.

These authors found considerable disagreement between the skin test and the complement fixation test using their antigen. The serological test in some cases became positive later in the disease than did the skin test. On the other hand, the complement fixation test remained positive in the terminal stages of the disease, whereas the animals became gradually anergic to the skin test. They comment:

We have found the most useful service of the test to indicate whether emaciated, scoursing animals, ones which usually failed to react to the allergic test, are affected with Johne's disease or with some other malady. For this purpose the test is probably the most reliable method available.
Hagan and Zeissig also obtained many "false positive" tests. They state:

In general it may be said that the serological test is frequently positive when the animal is certainly not affected with Johne's disease or tuberculosis, and there is not any apparent opportunity for sensitization of the animal with human or avian type tubercle bacilli.

They conclude from these experiments:

These animals interfere with the accuracy of the test to such a degree that it is not advisable to employ it in diagnostic work except to confirm the allergic test.

Sigurdsson (3, 29, 30, 31, 32) described a complement fixation test for paratuberculosis employing an antigen extracted from infected intestinal mucosa. The active antigen must be extracted from the mucosa by a special method. The following method of preparing the antigen has proved satisfactory:

Heavily infected intestinal mucosa is dried in a vacuum oven at about 45°C. The dried flakes are ground in a ball-mill for a specified length of time. The resulting light powder is suspended in saline in 3 per cent suspension, and the H·ion concentration adjusted to about pH 4.3 to pH 4.5. After half an hour at room temperature the suspension is centrifuged, the sediment resuspended in the same volume of saline, the H·ion concentration adjusted to pH 8.5 and the suspension boiled for one hour in a closed glass container. The suspension is then again centrifuged; the neutralized supernatant constitutes the active extract.

Simple saline extracts of the infected tissue are inactive in the complement fixation test. The process of drying and grinding is necessary for subsequently obtaining active extracts and the preliminary extraction at a low pH, where the antigen itself is insoluble, usually greatly enhances the activity of the subsequent final extracts. The rationale of the preliminary extraction at a low pH is that by this procedure an inhibiting substance or substances are removed from the preparation. This is clearly demonstrated by the fact that subsequent neutralization of these acid extracts and addition to the antigenic extracts in suitable dilution, greatly diminishes the complement binding activity of the antigen. It has not been possible to demonstrate a similar inhibitory activity in extracts prepared by an identical method from normal mucosa. This indicates that the second inhibiting substance is also a specific product of the infection.

This test has been used on a considerable scale in Iceland and has been found to be quite useful. Its main advantages compared with the skin test are that it generally becomes positive at an earlier stage of the infection, and further and less important, that it remains positive to the end, whereas, as already stated, most animals become anergic to the skin test in the last stages of the disease. Another important advantage of the complement fixation test, as employed in Iceland, is that false positive reactions are comparatively rare or approximately 2 per cent in healthy flocks of sheep. This compares favorably with the skin test. On the whole this complement fixation test has proved to be a valuable diagnostic tool.

Gislason (5) has shown recently (figure 4) that at least in certain populations strong reactions with this antigen are diagnostic, but the reliability of the test decreases as the reactions become weaker.

Hole (5, 33) has more recently revived the use of suspensions of artificially cultivated M. paratuberculosis as antigen in the complement fixation test. The technic of his test is unusual in that only the antigen (whole bacteria) and serum to be tested are mixed first. The sensitized bacteria are sedimented in the centrifuge, the supernatant serum dilution discarded, the sedimented bacteria resuspended in saline and only then is complement added. Hole reports that over 90 per cent of infected animals react in the test and that over
20 per cent of animals without lesions are also positive. He tested only one dilution of serum. Hole concludes from his experience that the test as carried out in his laboratory has great practical value.

Schaaf (5) has reported on the use of the complement fixation test in the Netherlands. As antigen, artificially cultivated \textit{M. paratuberculosis} were employed, but the technic of the test is along conventional lines. Out of 179 animals found to be infected on post-mortem examination, 152 (86 per cent) were positive in the complement fixation test; 19 (9 per cent) were doubtful, and 8 (5 per cent) were negative. The Dutch workers are satisfied with this test as a practical diagnostic procedure.

It will be clear from what has already been said that animals infected with \textit{M. paratuberculosis} usually contain antibodies, which may be demonstrated in the complement fixation test employing a variety of antigens. With the antigens employed so far, at least, it is not possible to distinguish between infection with \textit{M. paratuberculosis} and other mycobacteria. This disadvantage might be improved by employing purified preparations of antigen.

The antigens employed so far have contained components specific for the genus \textit{Mycobacterium}. The possibility that besides these "genus-specific" antigens species-specific components are also present, and that these could be isolated and used in tests distinguishing between infections with the various species of mycobacteria should certainly not be excluded. The purification of antigenic components from cultivated bacteria and from infected tissue should be worthwhile problems for future research. Unless such fractionation of the antigens can be accomplished, complement fixation tests will not distinguish clearly between infections with the different species of mycobacteria.

The question as to which of the antigens so far employed is the best one to use in a diagnostic complement fixation test for paratuberculosis is difficult to answer. The various antigens would have to be applied in parallel in strictly quantitative complement fixation tests on the same samples of sera from suspected animals. The animals should then be carefully autopsied as soon as possible after the blood is collected, as this is the most reliable means of arriving at a diagnosis. This could perhaps best be arranged by bleeding animals from suspected herds in the slaughterhouse and then carrying out a careful post-mortem examination. However, it would seem that the first step should be to seek to isolate and purify the antigens because it is a waste of effort to compare in detail impure preparations that, perhaps cannot be exactly reproduced.

With the antigen preparations now available it may be said that the practical usefulness of the complement fixation test in any given animal population depends not only on the accuracy of the test as such but also on the immunological experience of that population with antigenically similar materials presumably originating from related infections.

For example, obviously a "genus-specific" complement fixation test will be of less value for diagnosing paratuberculosis in populations latently or overtly afflicted with other species of pathogenic mycobacteria (\textit{M. tuberculosis} var. \textit{bovis}, \textit{M. avium}) than in populations free of other mycobacterial infections. It seems likely that saprophytic mycobacteria may under certain circumstances play a similar role. "False positive" reactions are bound to occur in such populations unless the serological test can be made species-specific by employing a purified species-specific antigen. In judging such "false positive" reactions, however, it is well to keep in mind the increasing evidence that latent infections with \textit{M. paratuberculosis} are much more common in many localities than had previously been suspected. This question was discussed briefly in the beginning of the present review.

Since the observation of Middlebrook and Duboe (34) that sheep erythrocytes will absorb certain specific components from appropriate extracts of tubercle bacilli and then become agglutinable by sera containing the corresponding specific antibody it has been demonstrated repeatedly (35, 36) that similar specific components may be extracted from \textit{M. paratuberculosis}. Sera from animals inoculated or infected with the organism will agglutinate such sensitized erythrocytes. Such tests do not distinguish very clearly between antibodies to the various types of mycobacteria. Serum from an animal infected with one type will generally agglutinate red cells which have adsorbed components from "heterologous" as well as "homologous" mycobacterial strains, although titers may vary. Cells
coated with components from one type of mycobacterium will similarly be agglutinated by antisera to "heterologous" as well as to "homologous" mycobacterial strains. These cross reactions detract from the value of such tests for diagnosis in the same way as do corresponding "cross reactions" in the complement fixation test.

The value of these hemagglutination tests compared with other diagnostic methods has not yet been adequately studied, but in preliminary work certain limitations in their usefulness appeared (30).

III. ATTEMPTS TO PROVOKE ACTIVE IMMUNITY AGAINST PARATUBERCULOSIS

There is reason to believe that only a small minority of animals which become infected with Mycobacterium paratuberculosis ever develop the clinical disease. This means that a majority of animals can develop effective resistance to the natural infection if conditions are favorable. It should be possible to produce active immunity artificially in an infection of this type. However, few attempts have been made to develop a vaccination procedure effective against paratuberculosis. The reason for this is undoubtedly the rather considerable difficulties involved in measuring acquired resistance to mycobacterial infections in general and to paratuberculosis in particular. Furthermore the earlier unsuccessful attempts to develop an effective vaccination against tuberculosis have probably rather discouraged later workers from similar attempts against other related diseases.

French workers have since 1924 practiced the inoculation of living virulent M. paratuberculosis, suspended in oil, subcutaneously in cattle with a view to protecting them against paratuberculosis (37). Vallé et al. reported in 1941 (38) that up to that time the method had been employed on more than 250,000 cattle.

For preparing this vaccine selected strains of M. paratuberculosis are grown on a fluid synthetic medium (5). It is specified that the strains should be nonvirulent, but the methods employed for routinely testing this have not been published. When the cultures are fully grown, the bacteria are filtered off. One gram wet culture is ground in a ball mill for 10 minutes and then 150 ml of liquid paraffin is added gradually and ground for an additional 20 minutes. After this, 2 g of powdered pumice stone is added and finally 150 ml of olive oil is added to the mixture which is then thoroughly mixed with an electric stirrer. All ingredients and apparatus have been sterilized beforehand. The dose of this vaccine is 1.5 ml subcutaneously.

The French authors state that this method is generally harmless and that it yields good protection against infection. They have reported that up to 1932 about 35,000 animals had been treated, and that 5650 animals on 133 farms previously heavily infected had been completely protected, and that on 20 other similar farms with 800 head of cattle, the method had been partially effective. The French authors call their method "prémunisation," not immunization, and they seem to be of the opinion that the efficacy of the procedure is due to a mild local infection with the living bacilli. They emphasize the need for carefully controlling the pathogenicity of the bacterial strains used for preparing the vaccine.

These important studies by the French workers are open to the criticism that unvaccinated control groups were apparently not included in their experiment. It is notoriously difficult to assess the usefulness of a vaccine against a disease of this type unless adequate control groups are kept in parallel with the treated animals. This is perhaps particularly true of paratuberculosis, because of its well known habit of lying latent in a herd, and then suddenly flaring up again. Taylor's criticism (1), therefore, seems appropriate; he remarks:

Vallée, Rinjard and Vallée in 1934 announced good results following the use of their vaccine upon many thousand animals in the field. But so far as the writer is aware there is little evidence based upon controlled experimental work which would enable a true assessment of the vaccine to be made beyond that published by Hagen in 1935.

Hagan in 1935 (39) vaccinated 10 calves with a vaccine of the French type: these calves and 10 similar unvaccinated control calves were then fed infective material. Four of the unvaccinated control animals died of paratuberculosis during the experimental period, but none of those that had been injected with the living vaccine died. However, some of the vaccinated animals were seriously affected with paratuberculosis when the experiment was terminated. On autopsy anatomical lesions of paratuberculosis and acid-fast
bacilli were found in 8 out of 10 vaccinated animals and in 7 out of 10 of the unvaccinated control group. It could be concluded from this small experiment that the vaccine had not prevented infection, when an inoculum, which was probably very heavy, was fed to the animal, but on the other hand it seemed that the disease was more severe in the unvaccinated group.

Doyle in 1945 (40) reported that no serious ill effects were observed in cattle after the subcutaneous inoculation of a living vaccine of the French type, and he felt that a controlled trial of the procedure devised by Vallée and Rinjard was indicated. Doyle has recently reported on extensive field trials of this vaccine in England (5). From these studies it seems obvious that the vaccine yielded protection, but the data presented so far do not permit a quantitative evaluation of the protective effect, since comparable control groups were not included.

Hole (5) has reported that animals vaccinated with the living vaccine do not develop complement-fixing antibody. This is surprising, particularly in view of the fact that killed vaccine, suspended in mineral oil alone, provokes a very strong and protracted antibody response (41). It would be interesting to know whether the resorbable olive oil included in the French type vaccine seriously alters the immunizing properties of the preparation.

Sigurdsson et al. (41, 42) reported on attempts to develop a vaccine from heat-killed *M. paratuberculosis*. The adjuvant effect of mineral oil, when used as suspending medium for mycobacteria was employed in this work. The vaccine was prepared as follows: Two old laboratory strains of *M. paratuberculosis* originally isolated from cattle were cultivated on a fluid synthetic medium. When full grown, the bacteria were filtered off and heated at 70 C on the water-bath for one hour and then dried in the vacuum oven at 45 C overnight. The dried bacteria were then suspended in mineral oil containing 0.5 per cent phenol. The concentration of bacteria was so adjusted that the desired quantity would be present in 1 ml of suspension. The bacterial suspension was injected subcutaneously into sheep. The dose of dry bacteria was varied over a wide range—from 0.37 to 300 mg *pro dosi*.

Infiltrates of considerable size developed at the site of injection and these persisted for months or years. These infiltrates were larger after the larger doses of bacteria, and after the largest doses they penetrated into the muscle and sometimes followed the lymph paths up to the internal iliac lymph glands on the injected side. At autopsy 20 months later the infiltrates were found to be composed of fibrocaseous tissue and to contain apparently intact acid-fast bacilli. All the animals developed skin sensitivity towards avian tuberculin. Following the smaller doses of vaccine this was somewhat inconstant in some of the animals.

As measured by the complement fixation test this type of vaccine elicited a powerful and protracted serological response in all animals. The titers were on the average 32 to 128 times higher than those found in natural cases of paratuberculosis and they were at a maximum about 4 to 6 months after the injection and then started to decline very gradually.

In a small field experiment on six infected farms this type of vaccine provoked adequate resistance to infection (43). During the experimental period, which lasted from 1 1/2 to 3 1/2 years, none of 289 vaccinated animals died from paratuberculosis, but 16 out of 206 unvaccinated control animals died from this cause. When the experiment was terminated and the surviving sheep were autopsied, suspected lesions of paratuberculosis were found in only 4 animals of the vaccinated group, or in 1.8 per cent, but in 21, or 10.8 per cent, of the unvaccinated controls.

Subsequent to this work another experiment on a larger scale was made (5). A total of 3073 sheep in a heavily infected area were vaccinated in the autumns of 1950 and 1951; 3184 sheep were left as unvaccinated controls on the same farms. This experiment is still in progress, but preliminary results obtained during the first 3 1/2 years confirm the results obtained in the first experiment; up to now 519 cases of paratuberculosis have occurred in the unvaccinated controls, as compared with 30 in the vaccinated group. It is clear that good resistance was produced in this experiment also. Figure 5 shows the results in one of eight groups in this experiment (5). In addition to the sheep in the field experiment in Iceland, about 190,000 sheep have been vaccinated with this new vaccine with apparently satisfactory results.

The question of cross-protection between infection with *M. paratuberculosis* and other
mycobacteria has not been extensively studied. Attempts have been made to learn whether inoculation with *M. tuberculosis* affords protection against a subsequent infection with *M. paratuberculosis* and vice versa or in other words whether cross-protection exists between these two infections. M'Tadyean and Sheather (44) obtained evidence on a small number of cattle that intravenous inoculation of *M. tuberculosis* afforded considerable protection against subsequent infection with *M. paratuberculosis*. Doyle (45) has demonstrated that goats will develop a considerable resistance to tuberculosis after the inoculation of living *M. paratuberculosis* suspended along with powdered pumice stone in liquid paraffin and olive oil. Evidence from the reviewer's laboratory (unpublished) indicates that killed *M. paratuberculosis* suspended in mineral oil may give to guinea pigs protection against tuberculosis comparable to that given by BCG.

The scanty available evidence thus indicates that cross-protection may exist between these two related infections. This is a question of considerable general interest and it is also worthy of consideration in connection with attempts to vaccinate against one or other of these diseases under natural conditions of infections.

REFERENCES


