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BY

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THE EFFECT OF AN INJECTION OF MALLEIN ON THE SERUM DIAGNOSIS OF GLANDERS.

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Since the discovery of the causal organism of glanders by Loeffler and Schutz in 1883, numerous attempts at an improvement in the methods of diagnosis have been made.

The only method originally available consisted in the isolation of the infective agent, and was confined to the demonstration and cultivation of the organism. Later it was found possible to reproduce the disease by inoculation of infected material into susceptible animals, and this method of diagnosis still persists, the guinea-pig being perhaps the most suitable animal for the purpose, and the characteristic reaction first described by Straus being regarded as diagnostic of the disease,

The most important agent, however, in the diagnosis of glanders was the biological product of the bacillus mallei, generally known as mallein. The method of preparation of this agent, and its efficacy are well known, and while numerous veterinarians on the Continent are dubious as to its general utility, clinicians in this country appear to have obtained more satisfactory results. It is certain that without the aid of mallein it would have been impossible to have reduced the occurrence of glanders to the state which now exists.

Based upon the ophthalmic reaction for tuberculosis, Frohner (11) carried out a number of experiments with precipitated mallein in the case of glandered horses and numerous healthy controls. As a result of his observations he came to the conclusion

* Read at the meeting of the Central V.S., held at 10 Red Lion Square, W.C,, on Thursday, April 2nd.

that the ophthalmic test was superior to either the subcutaneous or cutaneous reactions, and urged that it should become a routine method of diagnosis.

There are, however, some cases in which the efficiency of mallein as a diagnostic agent cannot be entirely relied upon, namely, those instances in which an atypical reaction is obtained on more than one occasion in the same animal. It is in such cases that the methods of serum diagnosis have been specially advocated. The most important of of these methods are the agglutination and complement binding tests.

The agglutination test as a means of diagnosing glanders was first suggested by M'Fadyean in 1896, but was not generally adopted until the method was perfected by Schutz and Miessner, whose results were published in 1905 (12). It has since been employed in practically every country where glanders exists, and ample opportunity has, therefore, been furnished for observing its practical diagnostic value.

While there is no doubt that the agglutination test is of great value in cases of recent infection and of a virulent type, the blood in such cases showing an agglutinating titre of 1-1000 or higher, experience has shown that in the case of chronic glanders an agglutinating titre as low as that of normal horses (1-400) is frequently encountered, while on the other hand cases have been recorded in which the agglutinating titre of normal serum has been found to be as high as 1-800 and further, where the curve has been followed for some time, variations have been exhibited in the titre of normal horses.

As a result of the important researches of Bordet and Gengou concerning the phenomenon of hæmolysis, and the subsequent investigations of Ehrlich, Morgenroth, Sachs and others, it was found to be possible to apply an even more delicate test to the diagnosis of glanders—the "Complement Fixation" test, which was first suggested in this connection by Schutz and Schubert (2) in 1909. Hutyra and Marek (4) concluded, as a result of numerous tests carried out by them in 1910, that "the diagnosis of glanders by the complement fixation test has already given such accurate results that it may be considered as the best method for the determination of this disease at the present time." In 1911 Moller and Eichhorn (3) published the results of an extensive series of experiments carried out by them,

in which the superiority of the complement fixation test over other known methods of diagnosis was amply demonstrated.

While nobody has apparently doubted the specificity of the methods of serum diagnosis for glanders when such constituted the primary test, yet recently it has been stated by various investigators that a previous injection of mallein rendered the serum tests unreliable, since as a result of the mallein injection, both agglutinating and complement binding antibodies were produced far in excess of those normally present in the blood of healthy horses. While it was well known subsequent to the work of Pokschischewsky and Fedorowsky (5) that an injection of mallein produced a marked increase in the production of these specific antibodies in animals affected with glanders, either by natural or artificial means, it was not recognised until recently that such a stimulation occurred in the case of non-infected animals.

In the latter case some doubt appears to exist concerning the time at which such a production of antibodies occurs, and the period over which the high titre is maintained. Bonome (6) was of opinion that the high agglutinating titre exhibited by such treated animals did not last for more than 5 to 7 days, and Arpad (7) showed that an agglutinating titre of 1-1200 to 1-1600 began to fall after 7 days. Miessner (8) found that the agglutinating titre began to rise in from 5 to 7 days after the injection of mallein, the maximum being attained towards the end of the second week, while the normal was regained in from 4 to 6 weeks. Arpad Marcis (9), as a result of experiments carried out on two animals, a stallion and a foal, came to the conclusion that after an injection of 5 cc. mallein there was a production of agglutinating and complement binding antibodies far in excess of the normal. He was able to demonstrate an increase in the former in from 5 to 7 days, and in the latter in from 6 to 10 days after the mallein injection, and found that the titre in each case began to fall in from 2 to 3 weeks, and in 3 months had regained the normal. Mohler and Eichhorn (10), on the other hand, are of the opinion that the immune bodies produced as a result of the stimulation of the mallein injection will have disappeared in from 7 to 10 days.

There seems, therefore, to be some doubt concerning the period over which this excess of immune bodies persists, and also the time at which the increase begins. The following experiments have been carried out with a view to acquiring a more exact knowledge of these facts.

The following is a brief description of the technique employed in carrying out the tests.

Technique of the Agglutination Test.

The antigen consisted of a saline suspension of killed B. mallei of a standard concentration, the organism being obtained from a 24 hours old surface culture on agar or blood agar.

The blood to be tested was obtained in the ordinary way from the jugular vein in the case of the larger animals, and from the ear veins in the case of the smaller laboratory animals.

The blood (2-3 c,c.) was collected in test tubes, which were placed in the incubator or water bath at 37° C. in a slanting position until the serum had separated off.

In the routine examination the following dilutions of serum were employed:—1 : 125, 1 : 200, 1 : 250, 1 : 400, 1 : 500, 1 : 750, and 1 : 1000.

If the blood from several animals was being tested, the pipette was thoroughly washed between each sample, and was always rinsed out with saline between each dilution, in order to avoid any abnormal results in the higher dilutions consequent upon a trace of serum having been left in the pipette.

The dilutions having been made, 1 c.c. of antigen was added to each tube, followed by 1 c.c. of the diluted serum. The tubes were then well shaken and incubated overnight at 37° C. In recording the results of the agglutination test, if the last tube containing 1/1000 c.c. of serum showed complete agglutination, the titre was said to be less than 1/2000. If the last tube showed nearly complete agglutination, the titre was recorded as equal to 1/2000. If the last tube showed only a trace of agglutination, while the next to the last showed complete clumping, the titre was said to be equal to 1/1500.

Technique of the Complement Fixation Test.

The following reagents were employed in the quantities stated :

(*a*) The serum to be tested.

(*b*) The complement 0·05 c.c., *i.e.* 0·5 c.c. of a 1 in 10 dilution.

(*c*) The antigen 0·5 c.c. of required concentration.

(*d*) The hæmolysin 0·001 c.c., *i.e.* 0·5 c.c. of a 1 in 500 dilution.

(*e*) The red cells (sheep) 0·5 c.c. of a 5 per cent. suspension.

The proportions recommended by Surface were also employed to control the results obtained with the above system, viz. :

(*a*)

(*b*) 1·5 times amount required to produce hæmolysis.

(*c*) As required.

(*d*) 0·25 c.c. of a 1 in 50 dilution (5 times amount required to produce hæmolysis).

(*e*) 0·5 c.c. of a 2 per cent. suspension.

(*a*) The serum was obtained in the same manner as for the agglutination test, and the following routine dilutions were employed : 0·3, 0·2, 0·1, 0·05, 0·02, 0·01.

(*b*) The complement consisted of fresh guinea-pig serum suitably diluted.

(*c*) The antigen consisted of either a saline suspension of B. mallei, which had been killed by heating for 1 hour at 60° C., or a solution of precipitated mallein, and in some cases the antigen was prepared by dissolving the bacterial cells in a 10 per cent. solution of anti-formin. The dissolved bacterial substance was then precipitated with alcohol, separated by centrifuging and suspended in saline solution. A fourth method of preparing the antigen was to *alternately* freeze and thaw a suspension of the organisms, the mixture being well shaken between each thawing and subsequent freezing.

The amount of antigen employed in each case was one-half the quantity required to produce self deviation, *i.e.* to absorb the amount of complement used.

(*d*) The hæmolysin was horse serum sensitised by sheeps red cells, and possessing a hæmolytic titre of 0·001.

(*e*) The second antigen—sheeps red cells were obtained in the ordinary way, and had been washed three times in normal saline solution.

All reagents were titrated before use.

A water bath at 40° C. was used in place of the thermostat at 37° C., a considerable saving in time being in this way effected, since the results could be read at the end of 20 minutes instead of one hour. Control experiments were also carried out, in which the water bath was used at 37° C. for one hour.

The test was put up in the ordinary manner. Into each of a number of clean bacteriological test tubes was placed 0·5 c.c. of a 0·9 per cent. saline solution, followed by the required amount of antigen (ascertained from the titration of the antigen). 0·5 c.c. of a 1/10 complement was then added, followed by varying dilutions of the serum which it was required to examine, and which had previously been inactivated by heating for 1 hour at 56° C.

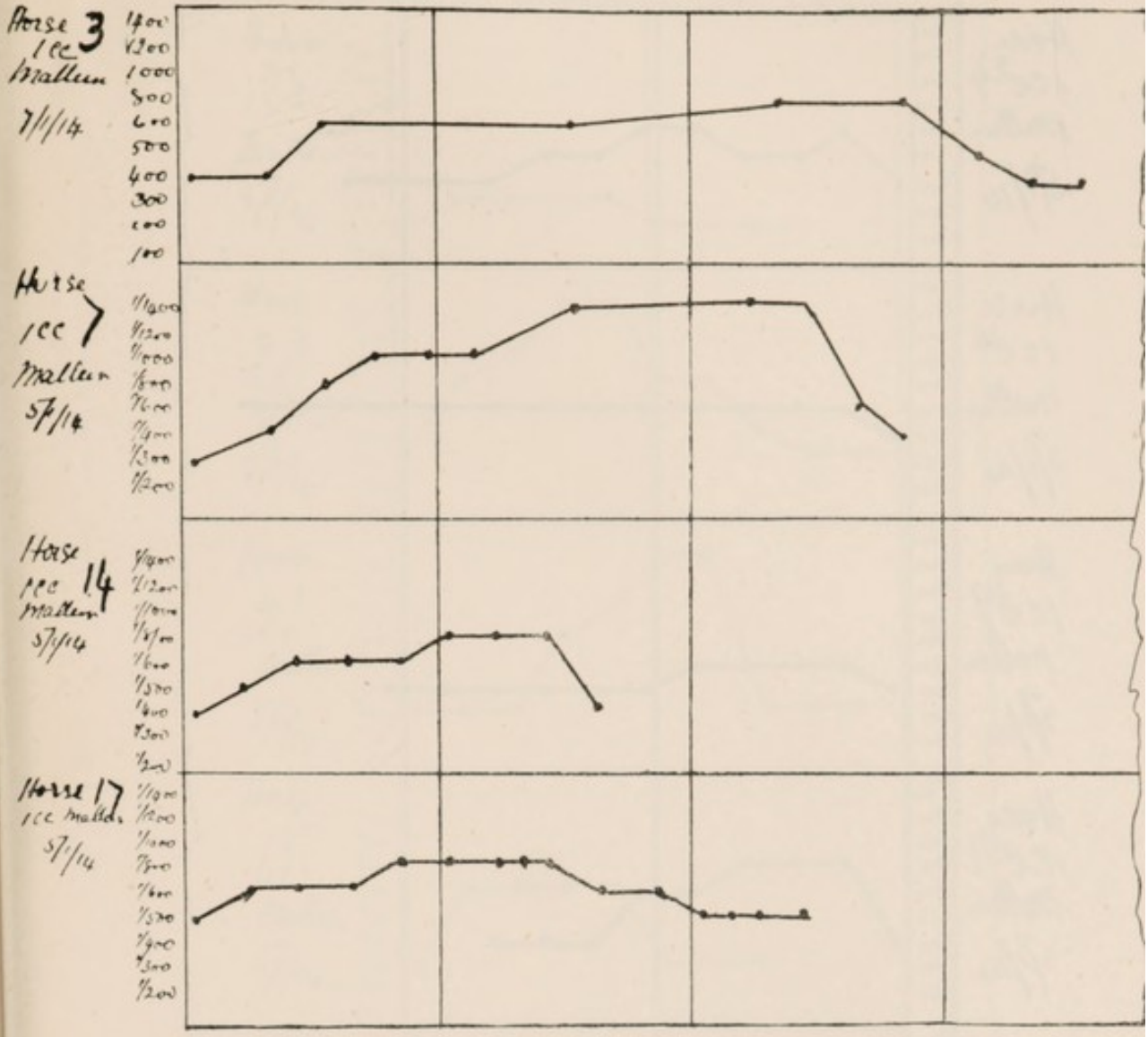
At the same time control tubes were put up containing all the constituents with the exception of the serum to be tested.

The tubes were then agitated to thoroughly mix the contents, and were left in the water bath at 40° C. for 20 minutes. At the end of that time, 0·5 c.c. of a 1/500 inactivated hæmolytic serum was added, and 0·5 c.c. of a 5 per cent. suspension of sheeps red cells. The tubes were again agitated, and left for a further 20 minutes, when the readings were taken. The highest dilution showing no hæmolysis was taken as representing the complement binding titre of that serum.

Agglutination Experiments.

In all 74 horses were used for these experiments. Of these 47 were being immunised against various bacteria or toxins, and with only two exceptions the results obtained with the blood of these horses were similar to those obtained with the blood of the remaining 27 normal horses. For the purpose of this paper it is sufficient if I confine my remarks to the results obtained with the latter. In all these experiments 1 c.c. mallein is equivalent to 0·1 c.c. concentrated mallein, and the normal agglutinating titre varied from 1/100 to 1/500.

Horse 3 received 1 c.c. mallein—normal titre 1/400. No rise was shown until the 6th day, when the titre was 1/600. From the 6th until the 23rd

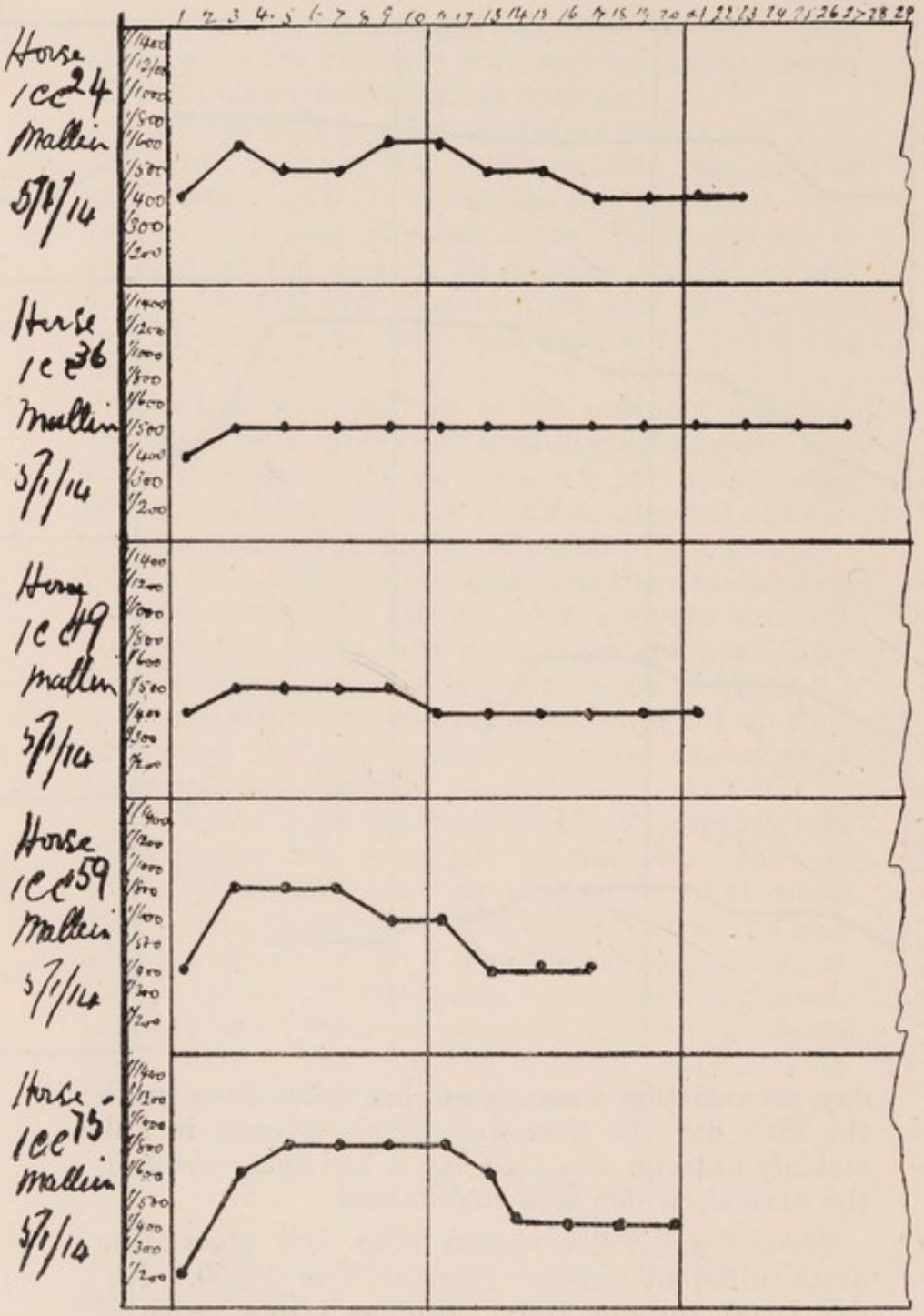


day, no variation was noticed, but from then until the 28th day the titre was 1/800, whence it fell steadily until on the 33rd day it had again reached the normal, which was maintained.

Horse 7 received a similar dose, but showed a quite different curve. Normal titre 1/300. 4th day 1/400, 6th day 1/800, 8th day 1/1000, with a steady rise until the 15th day, when it was found to be 1/1400. This titre was maintained until the 25th day, after which it fell steadily until the 29th day, when the normal was regained.

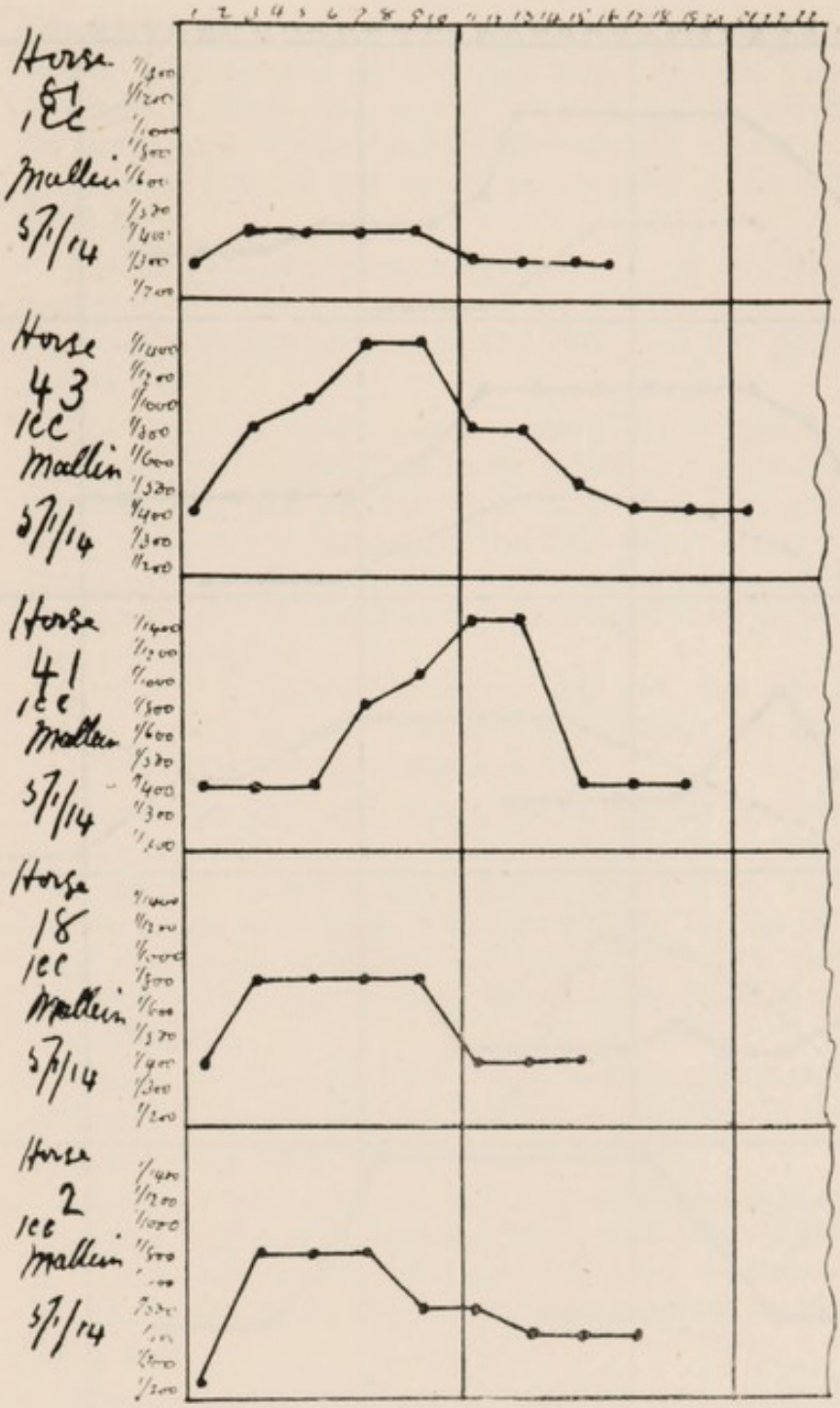
Horse 14 received 1 c.c. mallein. On the 5th day the titre had risen from 1/400 to 1/600; on the 11th day it had reached 1/800, where it remained until the 15th day, after which it fell sharply to 1/400 on the 17th day.

Horse 17. Normal titre 1/500, received 1 c.c. mallein. On the third day the titre was 1/600, the



next rise being on the 9th day, when it had reached 1/800. On the 16th day it had fallen to 1/600, and on the 21st day to 1/400.

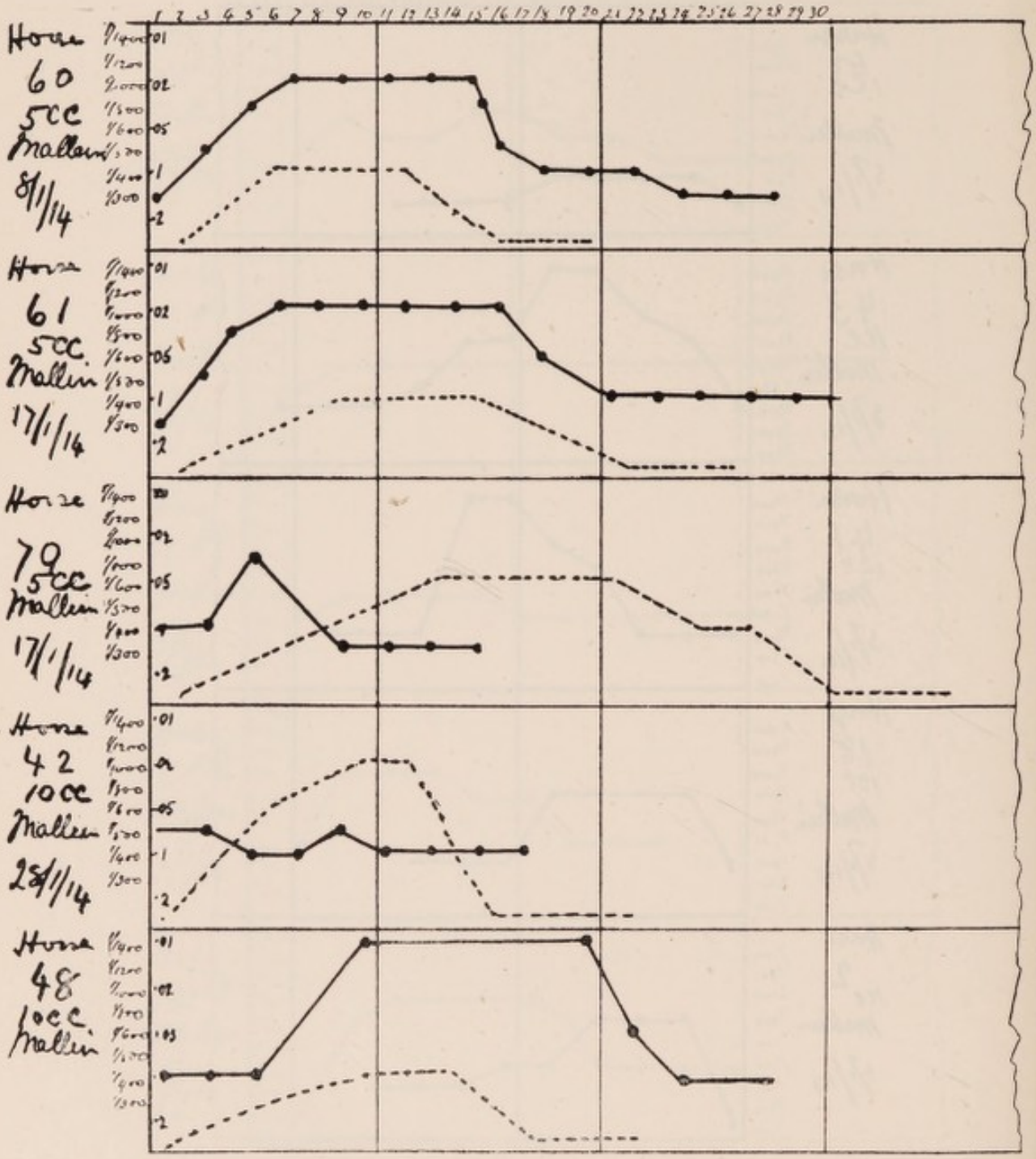
Horse 24. Normal titre 1/400, received 1 c.c. mallein, and showed a rather peculiar curve. On the 3rd day the titre had risen to 1/600; on the 5th it fell to 1/400—rose again on the 9th to 1/600, and fell again on the 13th to 1/400, which was maintained.



Horse 36. Normal titre 1/400, received a similar dose to the above. On the 3rd day the titre rose to 1/500, and remained constant for 27 days.

Horse 49 received 1 c.c. mallein. The titre rose from 1/400 to 1/500 on the 3rd day, and returned to 1/400 on the 11th day.

Horse 59. Titre rose from 1/400 to 1/800 on the 3rd day, fell to 1/600 on the 9th day, and to 1/400 on the 17th day.



Horse 75. 1 c.c. mallein. Titre rose from 1/200 to 1/800 on the 5th day, fell on the 11th day to 1/600, and on the 13th day to the normal.

Horse 81 received 1 c.c. mallein. The titre rose from 1/300 to 1/400 on the third day, remained constant until the 9th day, and again fell to 1/300.

Horses 43 and 41 each received 1 c.c. of mallein In the case of the former the titre rose sharply to 1/1400, which was reached on the 7th day, while that of horse 41 showed no rise until the 5th day,

when the titre began to rise sharply to 1/1400, which was reached on the 11th day. In the case of horse 41 there was a drop from 1/1400 to 1/400 in three days, while the titre of horse 43 fell more gradually, and did not reach the normal until the 17th day.

Horses 18 and 2 each received 1 c.c. of mallein, and in each case the titre rose to 1/800 only, and had regained the normal on the 11th and 9th days respectively.

Horses 60, 61, and 79 each received 5 c.c. mallein. The titre of the first rose from 1/300 to 1/1000 on the 7th day, began to fall from the 15th day, and had regained his normal by the 24th day. That of the second horse showed a very similar curve, but remained at 1/1000 for two days longer than in the case of horse 60.

The titre of horse 79 rose on the 3rd day from 1/400 to 1/600, and immediately began to fall, 1/300 being reached on the 9th day.

Horses 42 and 48. Received 10 c.c. of mallein each, and while in the case of the former there was no rise in the agglutinating titre shown, that of the latter rose from 1/400 to 1/1400 on the 5th day, remained constant until the 19th day, and fell sharply to the normal, which was reached on the 25th day.

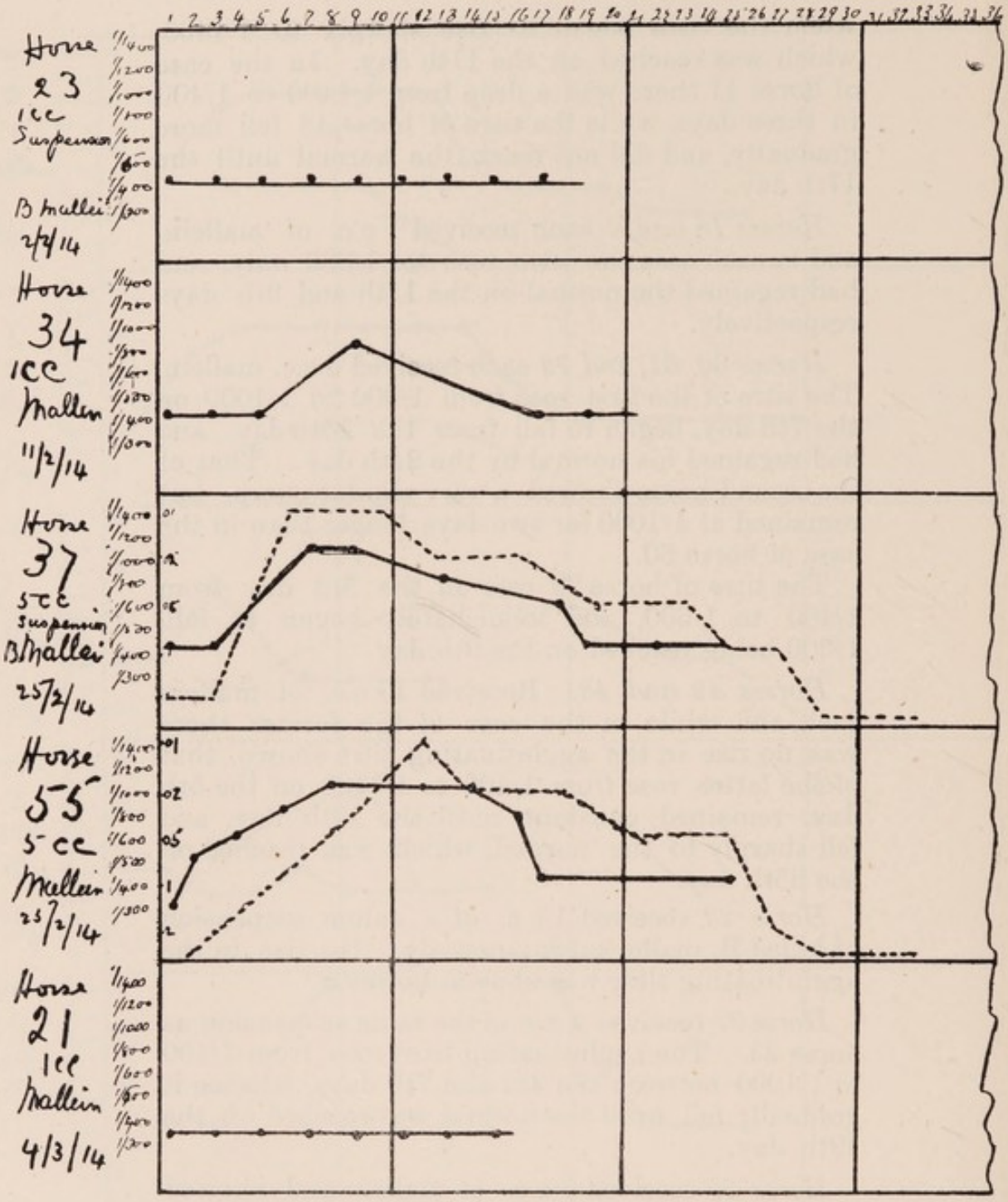
Horse 23 received 1 c.c. of a saline suspension of killed B. mallei subcutaneously. No rise in the agglutinating titre was shown, however.

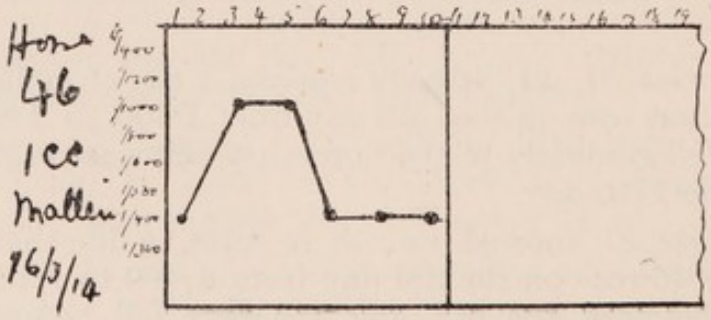
Horse 37 received 5 c.c. of the same suspension as horse 23. The agglutinating titre rose from 1/400 to 1/1000 between the 3rd and 7th days, whence it gradually fell, until the normal was reached on the 19th day.

Horse 55 received 5 c.c. of mallein and showed a curve which resembled very closely that of horse 37.

Horses 34, 21, 46 each received 1 c.c. of mallein. The first rose on the 5th day from 1/400 to 1/800, and fell gradually to the normal, which was reached on the 17th day.

Horse 21 showed no rise in titre, while that of horse 46 rose on the 3rd day from 1/400 to 1/1000, and between the 5th and 7th days fell again to the normal.


Horse 23 1cc Suspension B mallei 2/4/14
Horse 34 1cc Mallein 11/2/14
Horse 37 5cc Suspension B mallei 25/2/14
Horse 55 5cc Mallein 25/2/14
Horse 21 1cc Mallein 4/3/14


Horse 46 1cc Mallein 16/3/14

Horses 57, and *58* each received 1 c.c. of mallein. The normal agglutinating titres were 1/200 and 1/300 respectively, and no rise in titre was produced.

Horses 38 and 33 each received 5 c.c. of glycerine broth, there was, however, no increase in the agglutinins.

Two cows, 1 sheep, 1 goat, 1 dog, 4 rabbits, and 6 guinea-pigs each received 1 c.c. of mallein. In no instance, however, was it possible to demonstrate an agglutinating titre for B. mallei.

Two rabbits and 2 guinea-pigs infected with living B. mallei gave the following titres: 1/400 1/1600, 1/1000, and 1/400 respectively.

Complement Fixation Experiments.

The blood of all these horses was examined for complement binding properties on alternate days, beginning on the day following the injection of mallein.

Horses 3, 7, 14, 24, 36, 49, 59, 75, 81, 43, 41, 18, 2, 34, 21, 57, 58, and 46, all of which received 1 c.c. of mallein, at no time showed any complement binding titre to any of the antigens employed.

Horses 60, 61, 79, and 55, each of which received 5 c.c. of mallein gave complement binding titres of 0.1, 0.1, 0.05, 0.01 respectively, but only when mallein was used as the antigen. In no case was any titre shown with an antigen prepared from the actual organisms.

Similar results were obtained with the blood of *horses 42 and 48*, which received 10 c.c. of mallein each, the complement binding titre to mallein being 0.1 and 0.02 respectively.

Horse 23 which received 1 c.c. of a saline suspension of B. mallei did not show a titre with any of the antigens.

Horse 37, however, which received 5 c.c. of the above suspension gave a titre of 0.01 when mallein was employed as the antigen, but not in the presence of any preparation of B. mallei.

Horse 38 which received 5 c.c. of glycerine broth did not give any titre to either B. mallei, or mallein.

Neither the cow, sheep, goat nor dog gave any complement binding titre to any of the antigens

employed. The rabbits and guinea-pigs, however, which had received an injection of mallein showed in the presence of mallein as the antigen, titres varying from 0.1 to 0.02.

The two rabbits and two guinea-pigs suffering from glanders gave the following titres :—

		B. mallei			
		Suspension	Anti-formin	Frozen & thawed	Mallein
Rabbit	(*a*)	0.01	0.01	0.01	0.02
,,	(*b*)	0.01	0.01	0.02	0.02
Guinea-pig	(*a*)	0.05	0.05	0.05	0.05
,,	(*b*)	0.1	0.1	0.1	Nil

Summary.

Of the 27 normal horses used in these experiments, 19 received a subcutaneous injection of 1 c.c. of mallein. In three only was the agglutinating titre as high as 1/1400 ; in one it reached 1/1000 ; in eight 1/800 ; in one 1/600 ; in two 1/500 ; and four showed no rise in titre.

In the case of the 47 horses which were being immunised against various organisms or toxins, four only gave a titre of 1/1000 ; eleven gave 1/800 ; and the remaining 32 gave titres which varied from 1/300 to 1/600. Of the animals other than horses which received 1 c.c. of mallein, none showed any agglutinating titre to B. mallei, while the two rabbits and two guinea-pigs suffering from glanders gave titres varying from 1/400 to 1/1600 to that organism.

The 19 normal horses which received 1 c c. of mallein failed to show any complement binding titre in the presence of any of the antigens employed. The same result was obtained in the case of 45 of the 47 horses which were being immunised against various organisms or toxins. The two exceptions were horses being immunised against gonococcus and meningococcus, each of which gave a titre of 0.1 in the presence of B. mallei, but not with mallein as the antigen.

The four horses which received 5 c.c. of mallein, and the two which received 10 c.c. showed complement binding titres to mallein varying from 0.1 to 0.01. Those which received 10 c.c. did not show a higher titre than that which was produced in other horses which received 5 c.c.

In two horses which received 5 c.c. of glycerine broth did not show complement binding titres to either B. mallei or mallein.

While the blood of animals, other than horses, which had received 1 c.c. mallein showed no complement binding titre to any of the antigens employed, that of rabbits and guinea-pigs suffering from glanders gave titres varying from 0.1 to 0.01 with the various antigens, and in only one case was a negative result obtained.

A peculiar feature of these experiments was the fact that while the horse which received 1 c.c. of a saline suspension of killed B. mallei gave no complement binding titre—the blood of that which received 5 c.c. of suspension gave a marked titre to mallein, but none to B. mallei,

In neither the agglutination test nor the complement fixation test was it possible to fix definitely the time at which the titre rose and fell. In the former case it was found to begin to rise between the first and fifth day, and to fall between the fifth and 33rd day, while the complement binding titre began to rise from the first to the 14th day and to fall between the 14th and 31st day,

Conclusions.

From these experiments it would seem that a subcutaneous injection of 1 c.c. mallein is capable of stimulating in some horses the production of agglutinating antibodies specific for B. mallei, and that such a production may take place in from 24 hours to five days after such an injection, and may persist for 33 days, or even longer. If, therefore, it is required to carry out agglutination tests for glanders, the blood should be obtained either before the injection of mallein or not less than one month after.

Since the injection of 1 c.c. of mallein does not appear to stimulate the production of complement binding antibodies specific for B. mallei, there does not seem to be any objection to such a test being applied at any time irrespective of the period which may have elapsed since the injection of mallein.

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