THE CULTIVATION OF *SPIROCHAETA ICTEROHAEMORRHAGIAE* AND THE PRODUCTION OF A THERAPEUTIC ANTI-SPIROCHAETAL SERUM.

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(*Report to the Medical Research Committee.*)

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INTRODUCTION.

The Japanese investigators (Inada and Ido) who discovered the *Spirochaeta icterohaemorrhagiae*, the cause of infectious jaundice, showed that the serum of patients who had recovered from the disease contained immune substances which were capable of destroying the spirochaetes in the blood and tissues of an experimentally infected guinea-pig. They found that if the serum were injected into the guinea-pig prior to the appearance of icterus the disease was inhibited in all cases. The same result was obtained with the serum of immunised goats.

On the basis of this work Inada and Ido advanced the hypothesis that serum therapy should be effective in the human disease.

In the treatment of human cases they tried in the first instance the serum of recovered human cases and later the serum of actively immunised horses.

As the result of these first trials they came to the conclusion that the administration of the immune serum had a beneficial effect in cases of the disease.

Cases of jaundice with the clinical course of infectious jaundice were first observed among the troops on the Western front in the summer and autumn of 1915 but it was not until 1916, following the publication of the Japanese work, that *Spirochaeta icterohaemorrhagiae* was demonstrated in the blood and urine of the affected persons (Stokes and Ryle, 1916). The spirochaete was also found by Adrian Stokes (1917) in rats captured in the trenches where the cases of jaundice had originated.

In view of the favourable reports by the Japanese of the serum treatment of infectious jaundice and of the possibility of further cases arising among the British troops, seeing that the parasite was being harboured by the trench rat, it was decided by the Medical Research Committee to supply a curative serum for the treatment of cases of the disease.

The work of preparing the serum was entrusted to me and was begun in February 1917.

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I am indebted for my original material to Dr J. MacIntosh who sent me a portion of the liver of a guinea-pig which had died of experimental spirochaetosis. The strain of spirochaete used was that known as the Belgian strain and had been originally obtained by Captain Adrian Stokes from a case of infectious jaundice.

This strain had not at that time been brought into artificial cultivation and it was decided after discussing methods with Dr H. H. Dale to begin the immunisation of a horse with tissue spirochaetes and to continue with culture if cultivation experiments were successful.

My work on the subject therefore can be described under two heads dealing respectively with (*a*) the immunisation of horses with the spirochaete and (*b*) the artificial cultivation of the spirochaete.

It will be convenient to record first the cultivation experiments.

BIOLOGICAL CHARACTERISTICS.

Artificial cultivation of *Spirochaeta icterohaemorrhagiae*.

The Japanese observers, Inada and Ido, succeeded in cultivating *Spirochaeta icterohaemorrhagiae* by the method of Noguchi for the culture of the spirochaete of recurrent fever. I tried this method with various kinds of animal sera and human ascites fluid to which had been added, as recommended by Inada and Ido, guinea-pig kidney instead of rabbit kidney. The media were inoculated with pieces of the liver of an infected guinea-pig and were incubated at 37° C. and 25° C. both under aerobic and anaerobic conditions.

My experiments with these fluid media, like those of Adrian Stokes with similar media, were unsuccessful.

I then tried diluted bovine serum (serum 1 part and physiological salt solution 2 parts) which had been heated to 70° C. until it had become slightly viscous and I inoculated the tubes with the heart blood as well as with fragments of the liver of an infected guinea-pig. In this medium incubated at 25° C. a growth of the spirochaete was obtained at the first attempt and I was able subsequently to transmit the organism through several generations. Shortly after obtaining the first culture I had the opportunity, through the courtesy of the Secretary of the Medical Research Committee, of reading an advance proof of Noguchi's paper (1917) in which he reported the cultivation of the Belgian as well as the French and American strains of the spirochaete.

Noguchi grew the spirochaete in a fluid medium in which there were loose strands of fibrin produced by adding a small quantity of citrate plasma to the diluted or undiluted serum of a suitable animal. He recommended for routine use two media which had given equally satisfactory results:

(*a*) Rabbit serum (1 part) + Ringer's solution or 0·9 per cent. sodium chloride solution (3 parts) + citrate rabbit plasma (0·5 part) covered with a thin layer of sterile paraffin oil; (*b*) the same, except for the use of 0·5 to 1·0 part of neutral or slightly alkaline

agar (2 per cent.) which should be added while in a liquid state and quite hot (60–65° C.) in order to get a uniform mixture of the agar.

I was able to cultivate the spirochaete in these media but I obtained more luxuriant initial cultures in diluted serum which had been previously heated until it had become viscous or semi-gelatinous. This kind of medium was therefore adopted for the routine primary cultivation of the spirochaete.

The sera used were those of the horse, rabbit and adult cattle. Of these bovine serum has given the most consistently good results and has been most frequently employed. Bovine serum was also found by Martin, Pettit and Vaudremer (1917) to be very suitable for the cultivation of the spirochaete. Horse serum in a semi-gelatinous state proved less favourable for the growth of the spirochaete than semi-gelatinous bovine or rabbit serum, but horse serum unheated and mixed with 0·5 per cent. agar was found to be not inferior in nutritive value to bovine and rabbit serum.

Very satisfactory results have also been obtained with a medium composed of 1 part of the citrated blood of the horse, cow or rabbit mixed with 1 or 2 parts of physiological saline and heated until semi-gelatinous. In the horse blood medium the spirochaete sometimes grew extremely luxuriantly and one strain was transmitted through many subcultures in this medium. Ito and Matsuzaki (1916) recommended the use of a blood gelatin or a blood agar medium.

Noguchi (1918), in a recent paper, has recorded the results of his investigations of the value of different animal sera for the cultivation of the spirochaete. He found that the cultural value of different animal sera varies considerably. It is entirely absent from the sera of the rat and the pig. The sera of the rabbit, horse and goat are better suited for the growth of the organism than those of the guinea-pig, sheep, donkey or calf. Human serum is suitable but not ascites fluid. Fresh and heated emulsions of organs and the white and yolk of hens' eggs have no cultural value. (I also tried an egg medium, and, like Noguchi, failed to get a growth of the spirochaete.)

Noguchi found further that the nutritive value of serum is considerably reduced by heating to 60° C. for 30 minutes. My few comparative observations bearing on this latter point support Noguchi's finding. It has been observed that the addition of a small quantity of the citrated blood of a normal guinea-pig increases the nutritive value of a heated serum medium, thus indicating that the fresh blood restored to the medium something which was lost in the process of heating. This enrichment was specially noted with horse and rabbit serum. Bovine serum on the other hand, especially when of a golden yellow colour in the fluid state, showed little diminution in nutritive value after heating and produced luxuriant subcultures without any addition of fresh blood. With guinea-pig blood added semi-gelatinous bovine serum is in my experience an excellent medium for the cultivation of the spirochaete and by its use I have kept a strain of the organism in artificial cultivation for more than 18 months.

For raising initial cultures from an infected guinea-pig the citrated heart blood is the most convenient and suitable material. Liver and even kidney may be used but the cultures are more liable to become contaminated with other organisms than when blood is the material employed. Subcultures are made by transferring with a sterile pipette a few drops from the top of the medium to the surface of the fresh medium. All media are covered with a thin layer of sterile paraffin oil.

The spirochaete is an obligatory aerobe and grows best as Noguchi has also observed in the upper centimetre of the medium. When the medium is gelatinous spirochaetes are found only in small numbers in the middle and often not at all in the deep parts of the tube. In fluid media on the other hand there is wider distribution of the organism throughout the medium, possibly because oxygen, as Noguchi suggests, is able to penetrate more deeply into a fluid than into a semi-solid medium.

In semi-gelatinous media the spirochaetes are at first unevenly distributed in the upper layer. While some areas are colonised by enormous numbers of the organism others contain only moderate numbers and others again very few or none.

The spirochaete has been cultivated by me at two temperatures only, namely at 37° C. and 25° C. It is stated that it also grows at lower temperatures, down to 10° C.

Multiplication is as a rule more rapid at 37° C. than at 25 °C. but sometimes, in primary cultures, growth has been as rapid at 25° C. as at 37° C.

After two days' incubation at 37° C. (primary culture, semi-gelatinous serum medium) spirochaetes may be found in swarms in the upper centimetre of the medium. At the end of a week they are less numerous and degenerate forms are seen. In a fortnight they have diminished in numbers very considerably and there are many granules, the remains of degenerated spirochaetes. In the fourth week they have often disappeared completely from the medium which is no longer capable of infecting the guinea-pig. In order therefore to maintain a culture of the spirochaete at 37° C., subcultures should be made at short intervals. I did not however in my early experiments succeed in transmitting the spirochaete through more than five generations at this temperature and this result supported the statement of Inada and Ido (1916) that 37° C. is unfavourable for the cultivation of the organism. Recently I have had better success at this temperature, using a strain of the spirochaete which had been in cultivation for more than a year at 25° C.; this strain has been for several generations and is now growing luxuriantly at 37° C.

At 25° C. growth is in general not so rapid as at 37° C. but the organism retains its vitality for a longer period and can be subcultured through many generations. In subcultures at 25° C. spirochaetes do not as a rule become numerous until the second or the third week. When the medium is semi-gelatinous the spirochaetes begin after a time (two to three months) to degenerate and gradually to disappear while in more fluid media, though they

may diminish in number, they persist and remain viable for indefinite periods.

The Japanese observers, Inada and Ido (1916), stated that the life of a culture is variable.

> The first generation lived mostly from three to six weeks, the longest period observed being 55 days and the shortest 17 days. The life of the 2nd and 3rd generation is somewhat shorter than that of the first generation. The best time for transferring the culture from one tube to another is when multiplication is going on rapidly as indicated by examination of the fluid every two or three days.

My observations, while confirming the statement that the length of life of individual cultures varies, show that in a suitable medium the organism may live for very long periods not only in primary but also in secondary cultures.

Transference of the cultures have usually been made at intervals of from two to six weeks but subcultures up to 16 weeks old have grown luxuriantly in fresh tubes of medium. In fact there seems to be no limit to the age of a culture for successful subcultivation; if spirochaetes are present in the medium they will grow in a suitable new medium no matter what their age.

In order to ascertain how long a culture of the spirochaete would remain viable the first primary culture has been preserved (at 25° C.) and has been tested at intervals, the last test being made when the culture was 15 months old. On every occasion the spirochaetes were found to be capable of growing vigorously and luxuriantly in subculture.

No final statement therefore can be made at present as to the maximum duration of life of the spirochaete outside the body in a favourable medium. The primary culture is being preserved and will be tested again after a more prolonged interval.

PATHOGENICITY OF CULTURES.

Young recently isolated cultures of the spirochaete produce the same morbid effects in the guinea-pig as tissue spirochaetes. The pathogenicity of the cultivated spirochaete however appears quickly to be lost. A culture grown at 37° C., which, when a fortnight old produced typical spirochaetosis in a guinea-pig, lost its virulence within the next fortnight (two experiments). In another experiment with spirochaetes grown at 25° C., a 14 days' old primary culture produced fatal haemorrhagic jaundice while the same culture when $3\frac{1}{2}$ and 4 months old was completely non-pathogenic. A 14 days' old subculture from the four months' old primary culture was also devoid of virulence for the guinea-pig.

The guinea-pigs which failed to develop spirochaetosis after the injection of living cultures were after intervals of a month inoculated with emulsion of liver from a fatally infected guinea-pig. The guinea-pigs were entirely unaffected though control guinea-pigs receiving the same amounts of emulsion died in three to four days of acute spirochaetosis.

The above observations show that the virulence of the spirochaete for the guinea-pig is soon lost in culture and that the attenuated cultures of the organism are capable of inducing an active immunity in guinea-pigs.

Attempts to raise the virulence of the attenuated cultures by passage through the bodies of very young guinea-pigs, rats, mice and toads have not so far been successful.

The rats and mice were killed two to four weeks after intraperitoneal injection of the cultures and their kidneys were emulsified and injected into guinea-pigs. No spirochaetes were found in the emulsions, or in smear preparations of the spleen, liver and blood, and the guinea-pigs remained unaffected.

Toads (three experiments) were used with a view to ascertaining whether the spirochaete is capable of multiplying in the bodies of cold blooded animals. No spirochaetes were found in smear preparations of their organs, heart blood or subcutaneous lymph 14 and 28 days after inoculation.

The morphology of the spirochaete and the pathogenic effects in the guinea-pig.

My observations on the morphology of *Spirochaeta icterohaemorrhagiae* and on the gross pathological appearances of experimental spirochaetosis in the guinea-pig agree with those of other workers (Noguchi, 1918; Stokes and others, 1917; Inada and others, 1916) and I have nothing new to add to what has already been published in these connections.

The immunisation of horses.

Horse 1.

On 6 February, 1917, the immunisation of a horse was begun. As stated in the introduction it was decided to use as antigen in the first place emulsions of the livers of guinea-pigs which had died of experimental spirochaetosis and to continue with cultures if cultivation experiments were successful.

During the first four weeks the horse received seven injections of liver emulsions which had been sterilised by heat (at first 55° C. and subsequently 50° C.) or by the action of 0·5 per cent. phenol. The injections were made intramuscularly, the first dose being 5 c.c. of a thick emulsion, the seventh 20 c.c. containing the emulsifiable tissue of the livers of two young guinea-pigs.

After the latter injection the serum was found by Dr J. MacIntosh to have definite agglutinative and lytic action on living spirochaetes and it was considered therefore that the administration of the living organism might safely be begun.

The first dose of the living virus was 5 c.c. of liver emulsion rich in spirochaetes. The doses were thereafter gradually increased up to 25 c.c. containing the emulsifiable tissue of the livers of three guinea-pigs. As cultures of the spirochaete were now available the next dose (or tenth dose of living

virus) was partly liver emulsion and partly culture. Unfortunately only one guinea-pig was available and, as the cultures were only moderately luxuriant, this dose of antigen was therefore less rich in spirochaetes than the previous ones.

With a view to the speedy attainment of a high titred serum it was decided at this stage to continue the immunisation by the intravenous inoculation of cultures.

20 c.c. of a fluid culture containing numerous spirochaetes were accordingly injected into the jugular vein on June 15. Shortly after the injection the horse began to show signs of distress; then clonic contractions of the muscles set in and the horse fell down unconscious; respiration continued for a few moments when death ensued.

TESTS OF THE POTENCY OF THE SERUM.

The titre of the serum was determined at various stages of the immunisation by ascertaining the quantity of serum which would suffice to protect a guinea-pig weighing from 200 to 300 grams from a fatal dose of spirochaetes.

The test dose of virus was 1·0 c.c. of an emulsion of guinea-pig liver rich in spirochaetes. The doses were not standardised but the emulsions used were approximately of equal density. Though there was no doubt considerable variation in the numbers of spirochaetes injected in the different sets of experiments this variation did not appear to affect materially the comparative value of the tests.

The serum was injected a few minutes after the virus. Both virus and serum were injected intraperitoneally.

Three samples of the serum were tested. The first taken after about two months' immunisation gave a titre of 0·25 c.c., *i.e.* this amount of serum completely protected a guinea-pig from the test dose of spirochaetes. The second sample (May 11) had a titre of 0·1 c.c., while the third (June 14) was rather less potent than the second, 0·1 c.c. failing to protect the guinea-pig from death though it considerably prolonged the duration of life. The fall in the potency of the serum on June 14 is attributable to an insufficiency of antigen in the preceding dose (see above).

Horse 2.

The immunisation of a second horse was begun on June 18, 1917. During the first 18 days the horse received seven increasing doses of killed spirochaetes contained either in liver emulsions or in culture fluids.

A sample of serum taken five days after the seventh injection and 23 days after the beginning of the immunisation was tested for immune bodies.

First potency test.

Number of guinea-pig	Quantity of serum	Duration of life of guinea-pigs	Result
2 controls	—	Both dead, 4 days	Typical spirochaetosis
2469	1 c.c.	Died, 18 days	No haemorrhages or jaundice
2470	0·5 c.c.	Died, 15 days	Typical spirochaetosis
2471	0·25 c.c.	Died, 11 days	do.

This test showed that 1 c.c. of the serum was capable of protecting a guinea-pig from a dose of spirochaetes which killed controls in four days while smaller amounts of the serum prolonged the lives of the guinea-pigs.

The immunisation of the horse was continued with living spirochaetes derived either from cultures or from the livers of fatally infected guinea-pigs.

A second test of the serum was made after the sixth dose of living virus, 54 days from the beginning of immunisation.

Second potency test.

2 controls	—	Both dead, 4 days	Typical spirochaetosis
2482	0·5 c.c.	Died, 31 days	No sign of disease
2483	0·25 c.c.	Survived	
2484	0·125 c.c.	do.	

Another guinea-pig receiving 1 c.c. of the serum two days after the test dose of emulsion also survived.

This test showed that the serum had at least eight times the potency of the first sample.

As the doses increased in size it was found more convenient to use as principal antigen liver emulsion than culture. Liver emulsions contain enormous numbers of spirochaetes and to obtain approximately equal numbers in cultures would have required very considerable quantities of nutrient media. Whenever cultures were available however these were administered along with the emulsions. The largest dose of culture injected at one time was 30 cubic centimetres.

On September 18, eight days after the nineteenth dose of antigen and three months from the beginning of immunisation, the horse was bled 6 litres.

Third potency test.

Three sets of experiments were carried out with this serum. In the first two the smallest quantity of serum used was 0·05 c.c. and this in the two instances in which it was given afforded complete protection from a dose of spirochaetes which killed controls in from six to nine days.

A third series was done in order to ascertain the minimum quantity of serum which would protect, and the result is set out in the following table:

3 controls	—	All dead, 4 days	Typical spirochaetosis
2543	0·05 c.c.	Died, 104 days	No disease
2544	0·025 c.c.	Survived	—
2545	0·0125 c.c.	Died, 9 days	Typical spirochaetosis

On November 24, eight days after the 25th dose, which was 66 c.c. of an emulsion made from the livers of five guinea-pigs, the horse was bled 8 litres.

Fourth potency test.

2 controls	—	Died in 5 days	Typical spirochaetosis
2580	0·0125 c.c.	Survived	
2581	0·01 c.c.	Died, 6 days	No jaundice or haemorrhages
2582	0·01 c.c.*	Died, 5 days	
2583	0·006 c.c.*	Died, 6 days	No jaundice, slight haemorrhages
2584	0·005 c.c.*	Died, 6 days	Typical spirochaetosis

* Incubated with test dose 45 minutes.

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This test showed that since September 18 the serum had increased considerably in potency. 0·0125 c.c. now protected a guinea-pig completely from a dose which was fatal to controls in five days, while 0·01 c.c. was able apparently to destroy the spirochaetes in the test dose, though unable to save the lives of the guinea-pigs (microscopical examination of the liver of one of these latter guinea-pigs failed to reveal a spirochaete).

The immunisation of the horse was continued until the middle of March 1918, by which time 33 immunising doses had been given. On March 28 the animal was bled again. The potency of this batch of serum has not yet been tested because of the loss of the strain of spirochaetes which was being passed through guinea-pigs. Since January 1917 the strain had been passed from guinea-pig to guinea-pig until March 1918 when owing to a temporary shortage of young guinea-pigs adult guinea-pigs had to be used. The first of these died in seven days of spirochaetosis, but material from it failed to produce the disease in a second adult guinea-pig.

The first batch of serum sent out for the treatment of cases was from the first horse taken before the last and fatal dose of antigen. The titre of this serum was not very high (between 0·2 and 0·1 c.c.) but it was higher than that of the serum (titre 0·5 c.c.) used by the Japanese in their first series of cases. It was therefore supplied for trial in any case which might arise before a more potent serum became available.

The therapeutic results obtained by the Japanese workers (Inada, Ido, Hoki, Ito and Wani, 1916) with the low titred serum were very favourable. The serum considerably reduced the mortality rate and was found to be capable of destroying completely the spirochaetes in the circulating blood. Moreover the treatment promoted the development of antibodies and reduced the number of spirochaetes in the organs. At first they injected 10 c.c. of the serum into the subcutaneous tissues daily for three days but experience showed that this dosage was ineffectual. They therefore increased it gradually until finally they injected 60 c.c. in 24 hours. Later on they injected the serum intravenously and they found that this method far exceeded in potency subcutaneous injection. In a recent publication (1918) these observers give the results of the intravenous serotherapy of 41 cases of infectious jaundice. The spirochaetocidal titre of the sera used in this series was 0·01 and 0·03 c.c.

As a rule they injected 60 c.c. intravenously irrespective of severity of illness. Sometimes the entire quantity was given within 24 hours; at other times 40 c.c. in one day and 20 c.c. on the following day, or 20 c.c. on three successive days.

The action of the immune serum is spirochaetocidal and spirochaetolytic and best results are obtained when injections are made at an early stage of the disease. They found that intravenous injections are effective up to the fifth and sixth days of illness.

The total mortality figures, particularly of the severer cases, was considerably lower in the serum treated cases (intravenous and subcutaneous) than in the non-serum treated cases.

When the serum is administered early the disease appears to assume a milder form. The immune serum shortens the duration of the illness and has also a definitely beneficial influence upon haemorrhages, heart rhythm and suppurative processes.

The results obtained in this series led the authors to the conclusion that a titre of 0·01 c.c. suffices for the efficient serotherapy of infectious jaundice.

The November 24 serum from my second horse attained to this standard and should therefore be as effective in the treatment of the disease as the Japanese serum.

I have not yet any results to record of the therapeutic use of the serum.

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