LOUPING-ILL IN MONKEYS. INFECTION BY THE NOSE

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(With Plate V)

Louping-ill as is now known from the work carried out at the Moredun Institute, Edinburgh, during the last 10 years can be transmitted by the tick *Ixodes ricinus*. Under natural conditions the disease is probably most commonly spread by this means. The possibility that infection might result, at any rate under experimental conditions, by other means than the intervention of an arthropod vector has been the starting point of the following investigation. The nasopharynx as a possible portal of entry was considered first because it is so often incriminated in many of the virus diseases of man. Furthermore, Webster and Fite (1933) and Elford and Galloway (1933) have shown that mice can be infected with the virus of louping-ill by the nasal instillation of potent filtrates or suspensions of infective brain. Fresh importance has been added to this alternative route of entry of the virus by the occurrence of a number of cases of laboratory infections amongst those who have studied the experimental disease. Rivers and Schwentker (1934), who have recorded these cases, give reasons for incriminating the nose as the portal of entry.

Several of the larger domestic animals are known to be susceptible to the disease, and, in addition, Hurst (1931), Findlay (1932) and others have put on record the susceptibility of the monkey to direct cerebral inoculation of the virus. Two features which favour the monkey as the animal of choice are that it can be handled under ordinary laboratory conditions, and also that it shows extremely well that very characteristic destruction of the Purkinje cells of the cerebellum, a destruction which Hurst has shown to be far more extensive and regular in this experimental animal than in any of the others studied so far.

Methods

The rhesus monkey (*Macaca mulatta*) has been used exclusively in the course of this work.

The strain of virus used came originally from an experimentally infected sheep at the Moredun Research Institute. Since then it has been passaged in mice continuously and after forty such passages was still fully virulent for sheep (Elford and Galloway, 1933). The virus was administered in the form of a broth filtrate having a titre of about 1:100,000 and prepared from a 10 per cent. suspension of the brains of infected mice. The method of inocu-

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lation itself was to allow a few drops of the filtrate to fall into each nostril of the monkeys which were under light ether anaesthesia, the same procedure being repeated after an interval of 48 hours. The nasal mucosa of four of these animals was treated, prior to the introduction of the virus, with phosphate buffer *p*H 5·6 according to the method of Schultz and Gebhardt (1933), who claim that this preliminary treatment greatly increases the percentage of successes in experimental poliomyelitis; but the preliminary treatment with acid phosphate was discontinued since equally good results were obtained without it. It is even doubtful whether the double instillation of virus is necessary, since a positive result was obtained in each of two animals which received only one.

Out of a total of fourteen monkeys which received a broth filtrate of the virus into their nostrils, evidence of a successful infection was obtained in twelve. The tests for the presence of the virus in the various tissues or organs of these animals consisted of cerebral inoculations into each of three mice for each tissue. The results are given in detail in Table I.

Table I. *Results of nasal inoculation*

	Total	Results
Double inoculation and observed right through	6	5 positive, 1 immune
Double inoculation and killed on 5th day after the first	2	1 positive, 1 negative
Double inoculation and killed on 8th day after the first	2	1 positive, 1 negative
Double inoculation and killed on 12th day after the first	2	2 positive
Single inoculation and observed right through	2	2 positive

The term "positive" in Table I means that the animal was killed at the height of the disease at a time when it appeared likely to succumb to the infection. Recovery after the development of the characteristic symptoms of cerebellar involvement was observed in only one (No. 20) of the eleven monkeys described as positive. This animal was subsequently found to be resistant to a cerebral inoculation of the virus. In addition a twelfth animal (No. 16) showed no clinical symptoms of the disease, but neutralising antibodies to the virus were subsequently detected in its blood serum. Although the total number of animals used is small, the results demonstrate clearly that infection of the central nervous system can be readily obtained in the rhesus monkey by allowing a few drops of a broth filtrate of the virus to fall into its nostrils.

INCUBATION PERIOD

The incubation period of the clinical disease, *i.e.* the number of days which elapsed between the first inoculation and the appearance of symptoms referable to the central nervous system, averaged 17 days. The actual figures obtained in the seven animals concerned were 13, 14, 16, 17, 17, 20 and 22 days. After a cerebral inoculation of the virus in the same species of monkey, Hurst (1931) found the corresponding figure to be 8–9 days. We inoculated two monkeys intracerebrally, and they both developed the first symptoms on the 10th day.

Since the clinical symptoms of the disease, in which a cerebellar ataxia predominated, did not differ from those which Hurst observed after a cerebral inoculation of the virus, a description of them will be omitted here.

VIRUS CONTENT OF TISSUES AND INTERNAL ORGANS

Brain. In all the animals which were killed after the onset of symptoms, virus was recovered—as one might have expected from previous work on the properties of the virus—from the cerebellum and the medulla. Every part of the central or peripheral nervous system of some at least of these animals has proved infective for mice. The path followed by the virus in the central nervous system seems to be a steadily progressive one. The wide gaps devoid of virus which are found occasionally in experimental poliomyelitis, especially after an intrasciatic inoculation (Hurst, 1930)—the so-called axonal spread of the virus—was not observed in our animals. The gradual invasion of the central nervous system after a nasal inoculation is well exemplified by monkey No. 31, to which reference will be made further on.

Spinal cord and peripheral nerves. From a study of Table II it will be noticed that, provided the animal survives long enough, the virus will spread to the lower end of the spinal cord by the same gradual process which obtains in the brain. Similarly virus can be recovered from the peripheral nerves provided it has already reached the appropriate segment of the spinal cord. Thus, in monkey No. 33 where virus was recovered from the brachial nerves, but not from the sciatic nerves, the cervical and dorsal portions of the spinal cord were infective, but not the lumbar.

Infectivity of cerebrospinal fluid. The presence of virus in the cerebrospinal fluid was also studied in all but three of the monkeys, and virus was demonstrated in three of them. In monkey No. 30, which was killed on the 5th day after the first inoculation and whose blood contained virus, the sample of cerebrospinal fluid obtained on the 5th day was infective for mice but was blood-stained. The presence of virus in it therefore must be attributed to the admixture of blood. In monkey No. 33, killed on the 12th day after the first inoculation, virus was found in a sample of cerebrospinal fluid obtained on the 8th day and free from admixed blood, but none was found on the 5th or the 12th day—the only other occasions on which the cerebrospinal fluid of this animal was tested. In monkey No. 34 whose history is the same as that of No. 33 and whose blood was not infective, virus was found in cerebrospinal fluid on the 8th and 12th days, but not on the 5th day.

Blood and internal organs. A search was made for the presence of virus in the blood of several of our monkeys—in the case of two (Nos. 24 and 26) this was done every day until the development of symptoms. None was found except in the sample of blood obtained from No. 30 on the 5th day after the first of two inoculations, *i.e.* on the day on which it was killed. Certain organs of the same animal also proved infective, presumably owing to the presence of blood in them. These organs were the spleen, liver, kidney, lung and mesen-

Table II. *Presence of virus in the various tissues of the monkeys*

No. of monkey	29	30	31	32	33	34	21 A	21	17	24	15	
No. of days betwe first inoculation a death ...	5	5	8	8	12	12	15	16	16	17	25	
Olfactory lobe	0	0	+ + +	0	+ +	+ + +	0	0	–	+ + +	–	+
Frontal lobe	0	0	0	0	+ + +	+ + +	+ + +	+	+ + +	+ +	–	+
Parietal lobe	0	0	0	0	+ + +	+ + +	+ + +	+	+ + +	+ +	–	+
Temporo-sphenoid lobe	0	–	+ +	0	+ + +	+ + +	+ + +	–	–	+ + +	–	+
Occipital lobe	0	0	0	0	0	+ + +	+ +	0	0	+	–	+
Basal ganglia	0	0	0	0	+ + +	+ + +	+ + +	+ + +	+	+ + +	–	+
Hippocampus	0	–	0	0	+	–	+ +	0	–	+	–	
Pons and midbrain	0	0	0	0	+ + +	+ + +	+ + +	+ +	+ +	+ + +	0	+
Medulla	0	0	0	0	+ + +	+ + +	+ + +	+	+ + +	+ +	+ +	+
Cerebellum	0	0	0	0	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+
Spinal cord: cervic	0	0	0	0	+	0	+ +	0	+ + +	+ + +	+ + +	+
dorsa	0	0	0	0	+ +	0	0	+ +	+ + +	+ +	–	+
lumb	0	0	0	0	0	0	0	0	+ + +	+ + +	+ + +	+
sacral	0	0	0	0	0	0	0	0	+ + +	+ + +	+	+
Nerves: brachial	0	0	0	0	+	+	0	0	+ + +	+ +	–	
sciatic	0	0	0	0	0	0	0	0	+ + +	+ + +	–	
Pituitary	0	0	–	–	+	–	+ + +	0	+ + +	–	–	
Blood	0	+ + +	0	0	0	0	0	0	0	0	–	
Cerebrospinal fluid	0	+ (5)	0	0	+ (6)	+ + (6) + + + (12)	0	0	0	–	–	
Nasal mucosa	0	+	+ + +	0	0	0	0	0	0	0	–	

+ = virus present (the number of + signs shows number of mice reacting out of 3 inoculated).
0 = no virus found.
– = not tested.
Figures in brackets denote day on which virus was found.

teric lymphatic glands. In no other animal nasally infected were these or other internal organs found to be infective. It is unlikely therefore that normally the blood plays a part in the development of the disease after a nasal inoculation. The possibility of infecting the central nervous system of the monkey by an intravenous injection of virus was tested in two animals. They both received 5 c.c. of a filtrate which titred to 1 in 100,000 in mice. The blood of one (No. 79) was infective for mice for the first 6 days after inoculation, but not later. This animal developed no symptoms of the disease and remained alive and well for nearly 3 months. The other (No. 78) developed acute symptoms of incoordination, weakness and general collapse 48 hours after inoculation and was then killed, its blood being still infective for mice. Some internal organs (spleen, lungs and mesenteric glands) were also infective presumably owing to their blood content. No virus was detected in the central nervous system with the exception of one parietal lobe, where again its recovery might be explained by the presence of blood in some meningeal vessels attached to the piece of tissue used. No histological lesions which could be attributed to the action of the virus were detected in the cerebellum or elsewhere. In a third animal which received an intravenous injection of virus some 2 months after a sterile cerebral inoculation, typical louping-ill resulted on the 6th day. This indirect way of inoculating the brain—a procedure usually carried out in an inverse order to that used in this animal—is well known in other virus infections and differs entirely from the experiments just reported.

From two monkeys inoculated intracerebrally with the mouse virus samples of blood were obtained on alternate days until the development of symptoms on the 10th day and were tested on mice as usual. Virus was found on the 3rd and 5th day only in one animal, and on the 5th and 7th day only in the other. Following a cerebral inoculation therefore virus appears in the blood after an interval of 2–4 days and disappears again before the onset of symptoms. This is in marked contrast with what has been related above in the case of nasally infected animals where a direct spread to the central nervous system without the intervention of the blood seems to be the more usual occurrence.

The earlier stages of infection. The march of events in the development of the disease was studied in a few animals. Six monkeys received the double nasal inoculation at intervals of 48 hours, and they were killed in batches of two on the 5th, 8th and 12th day respectively after the first inoculation. In none of them had clinical symptoms yet developed. The presence of virus in the various organs and tissues was studied as before by cerebral inoculations into mice. The monkeys killed on the 12th day (Nos. 33 and 34) behaved like animals in which the disease had been allowed to develop and virus was found everywhere in the central and the peripheral nervous systems. In the case of the two animals killed on the 8th day no virus was detected in the tissues and organs of one, but some was demonstrated in the olfactory and temporosphenoidal lobes and at the site of inoculation (turbinates) of the other (No. 31).

In spite therefore of the great affinity of the virus for the medulla and cerebellum, it had not yet reached these areas, at any rate in demonstrable quantities, but it should be noted that although no inflammatory lesions were found in these areas, a small number of the Purkinje cells had disappeared. As regards the two 5-day animals, no virus was detected in the tissues of one (No. 29) but it was recovered from the blood, the cerebrospinal fluid and the turbinates of the second (No. 30). Various organs of this animal, though not the central or peripheral nervous systems, also contained virus, probably as the result of the presence of a good deal of blood in them.

Cellular changes in the cerebrospinal fluid. Samples of cerebrospinal fluid were obtained by cistern puncture in a number of our animals and in many on several occasions. It was found that as a rule only the first sample obtained from any animal by this method was free from admixed blood and so lent itself readily to cell counts, both total and differential. All these samples proved non-infective for mice. An average of one cell per c.mm. was found in all samples free from blood and obtained before the onset of symptoms. In those samples in which a pleocytosis was noted, lymphocytes were the only cells present. In one sample obtained on the first day of the clinical disease (No. 21) 1450 cells were counted per c.mm. In two samples obtained on the second day of the disease (Nos. 21A and 17) 360 and 560 cells per c.mm. respectively were found. On the 5th day after the onset of symptoms in a monkey (No. 20) which ultimately recovered, only 125 cells were counted.

On the whole therefore it may be said that a pleocytosis exists in the cerebrospinal fluid at the onset of the disease, and that the cellular exudate is entirely lymphocytic in character.

Proof of infection in recovered animals

It has already been stated that two monkeys survived the nasal instillation of virus. One (No. 20) developed clinical symptoms of the disease on the 17th day and ultimately recovered, the other (No. 16) remained free from symptoms for a period of some months. Neutralising antibodies were detected in the blood serum of both these animals, one month after inoculation in one (No. 16) and a similar period after recovery in the other (No. 20). Samples of serum which had been obtained from these two monkeys prior to inoculation, failed to neutralise the virus when tested on mice. In addition No. 20 received subsequently a cerebral inoculation of virus which it resisted without developing any symptoms.

Discussion

Attempts to infect sheep by the nasal route are recorded by Greig, Brownlee, Wilson and Gordon (1931), who used a suspension of infective brain for insufflation with a nebuliser in seven sheep. Of these one only developed mild nervous symptoms and recovered. Subsequently they were all found to be immune when tested by the cerebral route. Schwentker, Rivers and Finkel-

stein (1933) tried to infect four monkeys (*Macacus rhesus*) by repeated nasal instillation of a 15 per cent. suspension of mouse brain virus. No symptoms developed and subsequently they all succumbed to an intracerebral inoculation of the virus. Webster and Fite (1933) and Elford and Galloway (1933) had greater success with mice which they readily infected by the nasal instillation of suspensions or filtrates of mouse brain virus.

In the experiments recorded here clinical evidence of infection of the central nervous system was obtained in nine out of ten monkeys inoculated by the nasal route and kept for at least 12 days after inoculation. The use of a broth filtrate of the virus instead of a saline suspension of infective brain as in the experiments of Rivers, Schwentker and Finkelstein, and the fact that it was dropped into the nose under light ether anaesthesia afford the most likely explanation of the difference in results.

The fact that monkeys, mice, and probably sheep also, can be directly infected by the nasal route without the intervention of an arthropod vector gives additional support to the conclusion of Rivers and Schwentker (1934) who recorded a number of human laboratory infections, that the nose was the most likely portal of entry in their cases.

Gordon, Brownlee, Wilson and MacLeod (1932) have shown that the virus can be recovered from the blood of sheep after an intracerebral inoculation of the virus. The blood becomes infective on the 3rd day and continues so for a period of 48 hours approximately. Although in two of our monkeys inoculated intracerebrally we easily recovered the virus from the blood stream under similar conditions of time, we only once found virus in the blood of a nasally infected monkey, notwithstanding that samples of blood were obtained every day from two monkeys and tested on mice until typical symptoms of cerebellar involvement supervened.

Our own attempts to produce a central nervous affection in two normal monkeys by an intravenous injection of potent filtrates failed although the blood of these animals remained infective for several days after the inoculation. The positive result obtained in a third animal which had received a sterile cerebral inoculation some time previously is attributable to an indirect cerebral inoculation. We must therefore conclude that in monkeys, the virus follows a nervous path from the nose to the central nervous system, and further, that for reasons given earlier in this paper the spread of the virus throughout the brain and spinal cord is steadily progressive. Gordon (1934) states that in sheep the virus multiplies primarily in the blood and invades the central nervous system at a late stage in the infection. The results of the experiments recorded above do not suggest that there was any multiplication of the virus in the blood of our monkeys.

A general attempt was made to correlate the incidence of histological lesions with the presence of virus in the central and peripheral nervous systems of our animals. Since only adjacent pieces of tissue could be used for such a purpose, this correlation, although demonstrable in the majority of instances,

was not so uniform as might have been wished with the exception of the cerebellum where the presence of virus and of histological lesions always coincided. The character of the lesions in general did not differ from those previously recorded in the monkey (Hurst, 1931; Findlay, 1932). It is worth recording that the destruction of the Purkinje cells does not entail that of the basket fibres (see Plate V). A similar observation has been made in certain forms of cerebellar degeneration in man (*e.g.* Parker and Kernohan, 1933).

Conclusions

1. Rhesus monkeys can be regularly infected with the virus of louping-ill by allowing a few drops of a broth filtrate to fall into their nostrils under light ether anaesthesia.

2. The incubation period after a nasal instillation of virus varies from 13 to 22 days with an average of 17 days.

3. The virus can spread from the nose to the central nervous system without the intervention of the blood stream.

4. The spread of the virus through the central nervous system is steadily progressive from above downwards.

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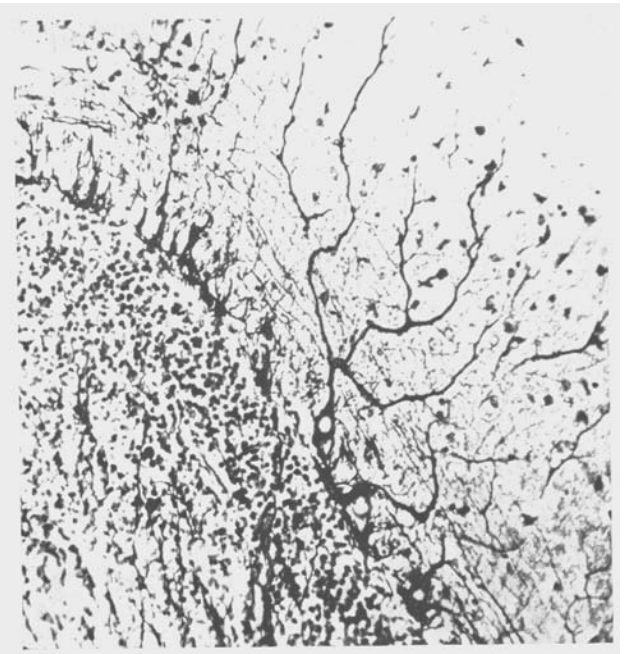

Section of cerebellum of monkey dying of louping-ill. Destruction of Purkinje cells with empty "baskets". Bielschowsky. Photomicrograph. × 185.

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