OBSERVATIONS ON THE IMMUNOLOGICAL RELATION OF POLIOMYELITIS TO LOUPING ILL

By F. F. SCHWENTKER, M.D., THOMAS M. RIVERS, M.D., AND M. H. FINKELSTEIN,* M.B., CH.B.

(From the Hospital of The Rockefeller Institute for Medical Research)

(Received for publication, March 2, 1933)

The fact that the etiological agent of louping ill is a filter-passing virus (1) capable of producing in monkeys and mice a disease somewhat similar to poliomyelitis induced us to determine whether any immunological relation exists between the maladies. The results of our investigations are presented in this communication.

Louping ill is a natural disease of sheep in Scotland and the northern part of England. The mass of literature which followed Duncan's original description of the malady in 1807 (2) has been fully reviewed by Pool (3) and indicates the serious nature of the scourge. Only lately, however, has the disease been separated from a number of other maladies giving rise to similar symptoms. The clinical manifestations of louping ill, tremors, hyperesthesia, ataxia, paresis, and paralysis, are caused by a disintegration of nerve cells among which the anterior horn cells of the cord and the Purkinje cells of the cerebellum are particularly involved. In addition to the destruction of neurons, a generalized hemic engorgement of the central nervous system and perivascular round cell infiltrations are usually observed. Louping ill has been transmitted experimentally to monkeys (4, 5), mice (1, 6, 4), swine (7), and probably to rats (1). Rabbits and guinea pigs, however, are apparently not susceptible (1). In general, the experimental disease is similar to the natural infection in sheep. In different hosts the symptoms are not necessarily identical, and such variations are probably occasioned by the type of nerve cell that is particularly involved. For instance, in monkeys, ataxia is the major symptom and is caused by a widespread disintegration of Purkinje cells in the cerebellum, while in mice, tremor, hyperesthesia, incoordination, weakness, and paralysis caused by a destruction of cells in the cerebrum and cord are the outstanding signs. Although no inclusion bodies have been found in the tissues of sheep, pigs, or monkeys, Hurst (4) has observed in the cytoplasm of nerve cells of mice acidophilic bodies which he believes belong to the general class of cytoplasmic inclusions.

* British Medical Research Council Fellow.

Hurst (4) has already pointed out that certain pathological findings in louping ill are similar to those seen in poliomyelitis, and this fact stimulated us to ascertain whether the maladies are immunologically related. Furthermore, the fact that vaccinia protects against smallpox made the problem more intriguing, because of the possibility that we might hit upon a simple method of immunization against poliomyelitis by the use of louping ill virus. With these ideas in mind we undertook the experiments reported at this time.

Methods and Materials

Louping Ill Virus.—The louping ill virus was procured from the Department of Bacteriology, University of Edinburgh, and was designated as follows: "obtained from the Moredun Institute, Animal Diseases Research Association, on 2nd February 1932 in the form of dried brain of sheep 43, 6th May 1931, since February 1932 maintained by intracerebral inoculation in mice." The virus was sent to us at The Rockefeller Institute in an infected mouse brain preserved in 50 per cent glycerol. Shortly after arrival, May 9, 1932, the material was washed several times in sterile Locke's solution and thoroughly ground in a mortar. Sufficient Locke's solution was then added to make a 10 per cent emulsion which was used to inoculate intracerebrally a group of mice and a monkey. After several intracerebral passages in monkeys and mice the brains of animals killed at the height of the disease were placed in 50 per cent glycerol under a vaseline seal and stored at 0°C. This material constituted the source of supply of virus for our experiments.

Poliomyelitis Virus.—The virus of poliomyelitis was supplied to us by Dr. Flexner in the form of a Berkefeld N filtrate of a 5 per cent emulsion prepared from the spinal cord of a monkey infected with the "mixed virus" strain.

Animals.—Only Indian monkeys (*Macacus rhesus*) were used in the experiments. The mice were of the Rockefeller Institute albino strain (*Mus musculus*).

Cultures.—The presence or absence of ordinary bacteria in infected organs was determined by means of aerobic and anaerobic cultures in meat infusion broth, pH 7.8.

Sections.—The clinical diagnosis of louping ill in each monkey was confirmed by means of histological studies of the central nervous system. In mice, however, histopathological examinations were made only when doubt arose as to the true nature of the illness. Tissues were fixed in Zenker's fluid or in a 10 per cent solution of formalin, sectioned, and stained according to Giemsa's method or by means of eosin and methylene blue.

Before undertaking the study of the immunological relation of poliomyelitis to louping ill it was essential for us to obtain monkeys immune to each of the maladies. Inasmuch as monkeys immune to poliomyeli-

tis were supplied by Dr. Flexner, it only remained for us to devise a safe method of immunizing monkeys against louping ill.

Immunization of Monkeys against Louping Ill

It is common knowledge among shepherds that sheep which survive a natural infection of louping ill are immune to the disease. Furthermore, Grieg, Brownlee, Wilson, and Gordon (1) have shown that sheep become resistant to louping ill following subcutaneous or intradermal injection, or intranasal insufflation of the virus. The last two methods are not without danger, however, since a certain number of the sheep develop symptoms of the disease during the process of immunization. Findlay (5) was able to immunize monkeys by intracerebral inoculation of sublethal doses of the infectious agent. The intracerebral method of immunization is obviously unsuitable for use in man. Furthermore, we were desirous of obtaining information regarding the production of disease of the central nervous system when the virus was introduced into the body in several different ways. Consequently, we attempted to immunize monkeys by intraperitoneal and intramuscular injections of virus and by intranasal instillation of the active agent.

Glycerolated infectious monkey brain was thoroughly ground in a mortar with alundum, and sufficient Locke's solution was then added to make a 10–15 per cent emulsion. After centrifugation at about 1000 R.P.M. for 5 minutes, the supernatant liquid was removed and used for the immunizing inoculations. A fresh emulsion was prepared for each set of inoculations and was tested for the presence of active virus by means of intracerebral inoculation of 0.03 cc. of the material into each of 6 mice. 16 monkeys received repeated inoculations of the active brain emulsions, and were then tested for immunity against louping ill. The results obtained are detailed below.

Intraperitoneal Injection.—Each of 8 monkeys received 3 intraperitoneal injections, 1 a week, of 1–5 cc. of a 15 per cent virus-brain emulsion. At no time did the monkeys have fever nor did they show signs of involvement of the central nervous system. 2 weeks after the last inoculation, the 8 treated and 6 untreated monkeys received intracerebrally 1 cc. each of a 10 per cent virus-brain emulsion. None of the immunized animals became sick, while all of the controls developed louping ill and died.

Intramuscular Injection.—Each of 4 monkeys received 3 intramuscular injections, 1 a week, of 3 cc. of a 15 per cent virus-brain emulsion. The animals remained well during the period of immunization. 2 weeks after the last inoculation,

the 4 treated and 2 untreated monkeys received intracerebrally 1 cc. each of a 10 per cent virus-brain emulsion. 3 of the treated monkeys developed typical louping ill, 1 on the 8th, 1 on the 10th, and 1 on the 11th day after injection. These animals were only moderately ataxic and gradually recovered. The 4th treated monkey showed symptoms of the disease on the 20th day after the intracerebral inoculation and was killed (it probably would have recovered) 3 days later for histological studies. The 2 control monkeys died of louping ill.

Intranasal Instillation.—Each of 4 monkeys received 3 intranasal instillations, 1 a week, of a 15 per cent virus-brain emulsion; 2 monkeys received 0.5 cc. amounts, the others 3 cc. amounts. None of the monkeys developed symptoms of louping ill. 2 weeks after the last instillation, the 4 treated and 2 untreated monkeys received intracerebrally 1 cc. each of a 10 per cent virus-brain emulsion. All of the animals developed louping ill and died.

It is obvious from a consideration of the experiments described above that the intraperitoneal injection of virus was the only method that completely protected the monkeys. Of 8 monkeys that were inoculated in this manner, none developed louping ill on subsequent intracerebral injection of an amount of virus sufficient to cause the disease in untreated animals. When immunization of the monkeys was attempted by instillation of the virus into the nares, no resistance was developed, and all 4 of the animals, when tested, developed typical louping ill. All of the monkeys that received immunizing injections intramuscularly also developed the disease when they were tested by intracerebral inoculation. 3, however, recovered almost completely and the condition of the 4th was such that death from the disease seemed unlikely when it was killed for pathological examination. In this animal, the incubation period was 20 instead of the usual 8 days. Since these were the only monkeys in our experience that recovered from the disease after signs had developed, it seems justifiable to conclude that they had acquired partial immunity due to the intramuscular injections of virus.

Inoculation of Louping Ill Immune Monkeys with the Virus of Poliomyelitis

As a result of our experiments on the immunization of monkeys against louping ill, animals refractory to that malady were available for inoculation with the virus of poliomyelitis. These monkeys, having been bled to secure serum for the neutralization experiments that will be described later, were handled in the following manner.

Each of 4 monkeys immune to louping ill and 2 normal monkeys (controls) received intracerebrally 0.8 cc. of Locke's solution plus 0.2 cc. of a Berkefeld N filtrate of a 5 per cent cord emulsion prepared from a monkey with poliomyelitis induced by the "mixed virus" strain. All of the animals developed poliomyelitis and died or were sacrificed when death was imminent. The louping ill immune monkeys and the control animals responded to the infection in a similar manner.

From the experiment just described, it seems evident that monkeys refractory to louping ill virus introduced intracerebrally are not immune to the virus of poliomyelitis administered in a similar manner.

Inoculation of Poliomyelitis Immune Monkeys with the Virus of Louping Ill

Monkeys that had recovered from attacks of poliomyelitis were bled to secure serum for neutralization experiments and then tested in the following manner for immunity to louping ill.

3 monkeys that had survived attacks of poliomyelitis induced by intracerebral or intracisternal injections of the "mixed virus" strain were supplied by Dr. Flexner. 2 of the animals had had the disease 17 months and 1, 10 months prior to the time they were received by us. The 3 poliomyelitis immune monkeys and 3 normal monkeys (controls) received intracerebrally 1 cc. each of a 10 per cent emulsion prepared from the brain of a monkey with louping ill. All of the animals developed louping ill and died. The monkeys immune to poliomyelitis and the control animals responded to the virus in a similar manner.

The above experiment indicates that monkeys which have recovered from attacks of poliomyelitis are not immune to louping ill virus introduced intracerebrally.

Neutralization Tests

In addition to the experiments already described it seemed advisable for us to pursue the investigation of the immunological relation of poliomyelitis to louping ill by testing the sera of monkeys immune to poliomyelitis for the presence of antibodies capable of inactivating the virus of louping ill. Before these experiments were undertaken, however, it was essential to determine whether such antibodies are present in the sera of louping ill immune monkeys.

The virus emulsions used in the neutralization experiments were prepared from pooled brains of mice killed at the height of a louping ill infection. After removal

from the animals, the brains were stored for 24 hours in separate containers in an ice box while bits of each were tested by means of cultures for the presence of ordinary bacteria. The brains free from bacteria were pooled and ground in a mortar. Sufficient Locke's solution was then added to make a 20 per cent emulsion which was centrifuged at 2000 R.P.M. for 3 minutes. The supernatant fluid was removed and again centrifuged. Then decimal dilutions of the supernatant fluid from the 2nd centrifugation were made with Locke's solution. Portions of each dilution were mixed with an equal amount of the serum, the neutralizing properties of which were being investigated. The mixtures were allowed to stand for 2 hours at room temperature and then for 1 hour in a refrigerator at 0°C. 0.03 cc. of each of the mixtures were then injected intracerebrally into each of 6 mice. The animals were observed for 18 days and the number of deaths in each group was recorded. No mouse that succumbed sooner than the 4th day after injection was considered to have died of louping ill.

In the manner described, an experiment was made in which serum from a louping ill immune monkey, serum from a normal monkey, and Locke's solution were each mixed with a set of decimal dilutions of louping ill virus. The results are summarized in Table I. Inasmuch as no deaths occurred in any group that received virus diluted more than 10^{-4}, the results with greater dilutions have been omitted from the death incidence calculations. Of the mice receiving virus mixed with normal monkey serum 70 per cent died, while among those that received virus plus louping ill immune serum only 21 per cent succumbed. The average time of death in the former group was 7.1, in the latter 9.4 days. From these results it appears that sera from monkeys that have recovered from louping ill are capable of neutralizing the virus. In the summary of the experiment shown in Table I, however, one finds that the incidence of death, 9 per cent, is peculiarly low in the mice that received virus dilutions that had been allowed to stand in contact with Locke's solution instead of serum. Since it is highly improbable that Locke's solution contains specific neutralizing antibodies, it seemed likely to us that the virus was damaged by a toxic action of the Locke's solution. Accordingly an experiment was undertaken to determine whether this actually occurred.

Decimal dilutions of a virus emulsion were mixed with equal volumes of Locke's solution. 0.03 cc. of each of these mixtures were immediately injected intracerebrally into each of 6 mice. The remaining portions of the mixtures were

allowed to stand 2 hours at room temperature and 1 hour in a refrigerator at 0°C. After the period of standing, 0.03 cc. of the different mixtures were injected into each of 6 mice. The results appear in Table II.

TABLE I

Summary of Experiment to Determine whether the Sera from Monkeys Immunized against Louping Ill Contain Neutralizing Antibodies

Dilution of virus	No. of mice inoculated	No. of deaths	Percentage of deaths	Day of death	Average time of death
Louping ill immune serum plus virus dilutions					
10^{-1}	6	3	50	8, 8, 10	
10^{-2}	6	0	0		
10^{-3}	6	1	15	11	
10^{-4}	6	1	15	10	
10^{-5}	*6*	*0*	*0*		
	24	5	21		9.4 days
Normal monkey serum plus virus dilutions					
10^{-1}	6	6	100	6, 6, 6, 6, 7, 7	
10^{-2}	6	5	80	7, 7, 7, 7, 7	
10^{-3}	6	5	80	7, 7, 7, 7, 11	
10^{-4}	5	0	0		
10^{-5}	*5*	*0*	*0*		
	23	16	70		7.1 days
Locke's solution plus virus dilutions					
10^{-1}	6	2	35	7, 14	
10^{-2}	6	0	0		
10^{-3}	5	0	0		
10^{-4}	6	0	0		
10^{-5}	*6*	*0*	*0*		
	23	2	9		10.5 days

Figures in italics have been omitted from calculations.

The results of the experiment summarized in Table II show that of the mice that received the fresh mixtures of virus and Locke's solution 70 per cent died (average time of death, 6.7 days), while of the mice

that received the mixtures allowed to stand for 3 hours only 30 per cent died (average time of death, 10 days). Such results are similar to those obtained when dilutions of herpetic virus or the virus of yellow fever are allowed to stand in contact with physiological salt solution, and have been attributed to the toxicity of the diluting solution. Consequently, in subsequent neutralization experiments, normal monkey serum instead of Locke's solution was used as a control diluent.

TABLE II

Summary of Experiment to Show the Toxic Action of Locke's Solution on Louping Ill Virus

Dilution of virus	No. of mice inoculated	No. of deaths	Percentage of deaths	Day of death	Average time of death
Virus dilutions made with Locke's solution injected immediately					
10^{-1}	6	4	65	5, 7, 8, 8	
10^{-2}	5	4	80	5, 6, 6, 7	
10^{-3}	5	3	60	7, 7, 8	
	16	11	70		6.7 days
Virus dilutions injected after standing for 3 hrs. in contact with Locke's solution					
10^{-1}	5	1	20	7	
10^{-2}	5	1	20	8	
10^{-3}	6	2	35	6, 14	
	16	4	25		8.7 days

Having found that sera obtained from monkeys immune to louping ill contain antibodies capable of neutralizing the virus, we proceeded to determine whether such antibodies are present in the sera of monkeys that had recovered from poliomyelitis. 2 experiments in which louping ill immune serum, poliomyelitis immune serum, and normal serum were mixed respectively with decimal dilutions of louping ill virus and handled as described above were performed. The findings have been brought together in Tables III and IV. The results recorded in the former table show that none of the mice that received mixtures of

louping ill virus and louping ill immune serum died, while there was a mortality of 53 and 55 per cent respectively among the animals receiving mixtures of virus and normal serum, and virus and poliomyelitis

TABLE III

Summary of an Experiment to Determine whether Sera from Monkeys Recovered from Poliomyelitis Are Capable of Neutralizing Louping Ill Virus

Dilution of virus	No. of mice inoculated	No. of deaths	Percentage of deaths	Day of death	Average time of death
Normal monkey serum plus virus dilutions					
10^{-1}	5	5	100	6, 7, 7, 7, 7	
10^{-2}	6	2	35	7, 9	
10^{-3}	6	2	35	7, 10	
10^{-4}	*6*	*0*	*0*		
10^{-5}	*6*	*0*	*0*		
	17	9	53		7.4 days
Poliomyelitis immune serum plus virus dilutions					
10^{-1}	6	4	70	7, 7, 7, 8	
10^{-2}	6	4	70	7, 7, 8, 10	
10^{-3}	6	2	35	9, 14	
10^{-4}	*6*	*0*	*0*		
10^{-5}	*6*	*0*	*0*		
	18	10	55		8.4 days
Louping ill immune serum plus virus dilutions					
10^{-1}	6	0	0		
10^{-2}	5	0	0		
10^{-3}	6	0	0		
10^{-4}	*6*	*0*	*0*		
10^{-5}	*6*	*0*	*0*		
	17	0	0		

Figures in italics have been omitted from calculations.

immune serum. The findings displayed in Table IV are similar to these just enumerated: 77 and 70 per cent respectively of the mice that received mixtures of virus and normal serum, and virus and poliomye-

litis serum died (average time of death after inoculation, 6.7 and 7.1 days), while only 22 per cent of the animals that received mixtures of virus and louping ill immune serum succumbed (average time of death after inoculation, 10.2 days). Such results substantiate our earlier

TABLE IV

Summary of an Experiment to Determine whether Sera from Monkeys Recovered from Poliomyelitis Are Capable of Neutralizing Louping Ill Virus

Dilution of virus	No. of mice inoculated	No. of deaths	Percentage of deaths	Day of death	Average time of death
Normal monkey serum plus virus dilutions					
10^{-1}	5	4	80	6, 7, 8, 8	
10^{-2}	5	4	80	6, 6, 6, 6	
10^{-3}	6	6	100	6, 6, 6, 6, 7, 9	
10^{-4}	6	3	50	6, 7, 8	
	22	17	77		6.7 days
Poliomyelitis immune serum plus virus dilutions					
10^{-1}	6	5	85	6, 6, 6, 7, 9	
10^{-2}	6	5	85	6, 6, 7, 8, 9	
10^{-3}	6	2	35	6, 6	
10^{-4}	5	4	80	7, 7, 8, 9	
	23	16	70		7.1 days
Louping ill immune serum plus virus dilutions					
10^{-1}	6	4	65	9, 10, 11, 11	
10^{-2}	6	0	0		
10^{-4}	6	0	0		
	18	4	22		10.2 days

findings in regard to the presence of neutralizing antibodies in louping ill immune serum and also clearly indicate that no such antibodies for louping ill virus are demonstrable in the sera of monkeys that have recovered from poliomyelitis.

SUMMARY

The results of the work presented in the present paper show that louping ill and poliomyelitis immunologically are not closely related. Although relatively few experiments were performed, the data obtained were sufficiently decisive for our purposes. Certainly nothing was found to indicate that one might be able to immunize human beings against poliomyelitis by the use of louping ill virus. In addition to the negative findings, a certain amount of useful information was also secured, namely, (1) monkeys can be solidly immunized against louping ill by intraperitoneal injections of virus and partially protected by intramuscular administrations of the active agent, (2) during the process of immunization no signs of involvement of the central nervous system are manifested, and (3) sera from monkeys immunized in the manner described contain antibodies capable of neutralizing the virus.

REFERENCES

1. Grieg, J. R., Brownlee, A., Wilson, D. R., and Gordon, W. S., *Vet. Rec.*, 1931, **11**, 325.
2. Duncan, A., *Tr. Highland Soc. Scotland*, 1807, **3**, 339.
3. Pool, W. A., *Vet. J.*, 1931, **87**, 177, 239.
4. Hurst, E. W., *J. Comp. Path. and Therap.*, 1931, **44**, 231.
5. Findlay, G. M., *Brit. J. Exp. Path.*, 1932, **13**, 230.
6. Alston, J. M., and Gibson, H. J., *Brit. J. Exp. Path.*, 1931, **12**, 82.
7. Pool, W. A., Brownlee, A., and Wilson, D. R., *J. Comp. Path. and Therap.*, 1930, **43**, 253.