Acta vet. scand. 1980, *21*, 113—123.

From the Department of Microbiology and Epizootology, College of Veterinary Medicine, and the State Veterinary Medical Institute, Helsinki, Finland.

EXPERIMENTAL MAEDI INFECTION IN SHEEP

1. DETECTION OF VIRUS, CLINICAL COURSE, HISTOPATHOLOGY*

By

Liisa Sihvonen, T. Estola and *J. Tuomi*

LIISA SIHVONEN, T. ESTOLA and J. TUOMI: *Experimental maedi infection in sheep. 1. Detection of virus, clinical course, histopathology.* Acta vet. scand. 1980, *21*, 113—123. — Eight sheep were inoculated with Icelandic maedi strain M 88; 2 sheep served as control sheep and were in close contact with the inoculated ones. Four of the sheep were inoculated via the respiratory tract with 7×10^6 TCID50 of strain M88 and the other 4 intracerebrally with 5×10^5 TCID50 of the same strain.

Maedi M88 strain was isolated from peripheral blood leukocytes of all inoculated sheep. There was a striking difference between the 2 groups in the appearance of demonstrable viremia after inoculation. Viremia could be demonstrated in the intrapulmonarily inoculated sheep within 2—6 months but not until 8—11 months after inoculation in the intracerebrally inoculated ones. This finding is thought most probably to reflect a weak neurotropism of the strain used. After the first demonstration of viremia, maedi virus has been recovered quite regularly in peripheral leukocytes of all intrapulmonarily inoculated sheep, but less regularly in the intracerebrally inoculated ones. Maedi virus was isolated from 1 of the uninoculated control sheep 15 months after inoculation.

The first clinical case with a clinical appearance suggesting combined involvement of maedi and visna was found among the intrapulmonarily inoculated sheep, 8½ months after inoculation. Histopathological examination and virus isolation confirmed maedi. The cause of paraplegia could not be confirmed. No histopathological changes were found and no virus isolation was made from the central nervous system of this animal.

One of the intracerebrally inoculated sheep died suddenly without any observed clinical signs 11 months after inoculation. Histopathological examination revealed pulmonary lesions of maedi, but no visna lesions in the central nervous system, although maedi virus was isolated from various parts of brain.

None of the other experimental sheep displayed clinical signs of maedi or visna during the observation period of 18 months.

maedi virus; slow viruses; viremia; clinical signs; histopathology.

* This work was supported by the Finnish Academy.

Visna and maedi are slow virus diseases of sheep with long incubation period, and they are considered to be different manifestations of infection with the same ovine retrovirus (*Harter et al.* 1973, *Haase* 1975, *Pálsson* 1976). Maedi, a progressive interstitial pneumonia, is manifested as severe weight loss and chronic dyspnea often seen first during exercise. Clinical signs of maedi appear in adult sheep only, and death occurs several months to 1 year later (*Pálsson*).

Visna is progressive viral meningoencephalitis, and this clinical illness is not found in the field in any sheep younger than 2 years. The clinical course of visna is variable, but it terminates in paraplegia or total paralysis (*Gudnadottir* 1974).

The etiologic role of virus in visna and maedi was established through experimental reproduction of the disease in previously healthy sheep by contact exposure, by inoculation with suspensions of brain or lung from diseased sheep and by inoculation with virus grown in cell culture. In the field, infections are mainly transmitted by the respiratory route in the form of droplet spread of the virus, which favors pulmonary involvement (*Pálsson*).

Visna and maedi have been observed in sheep in many countries, among them in all Nordic countries, Finland excepted (*Mårtensson et al.* 1975).

In this first paper of a series of reports presence of virus and the manifestations of its pathogenicity in sheep inoculated differently are presented.

MATERIALS AND METHODS

Cell cultures

Cell cultures were established from normal 6—12 months old Finnish sheep. Choroid plexus was taken from the brains, minced and explanted into 25 cm^2 plastic flasks (Falcon), followed by growing in Eagle's minimum essential medium (MEM, Orion) plus 20 % fetal calf serum (FCS). Sheep choroid plexus (SCP) cells were then trypsinized and passed into 75 cm^2 plastic flasks (Falcon). At confluence the cells were again trypsinized and resuspended in MEM plus 20 % FCS and 10 % dimethylsulfoxide and divided into ampules, which were stored in liquid nitrogen. Cells from each ampule were used for 15—18 subcultures. These cells were grown in MEM plus 15 % FCS and maintained in MEM plus 2 % FCS. All cell cultures used for virus isolation, viral

assay or serum neutralization tests were washed twice before inoculation and maintained with MEM plus 2 % lamb serum (LS), because FCS inhibits the replication of visna-maedi virus (*Thormar et al.* 1962).

Virus

Maedi strain M88 isolated from maedi-affected lungs in 1961 (*Gudnadottir & Pálsson* 1967) in Iceland and passed many times in SCP cells since isolation was kindly provided by Dr. G. Petursson of the Institute for Experimental Pathology, Iceland. In Finland, the M88 strain was first passed in SCP cells 4 times. The cell culture fluid of the fourth passage was centrifuged at 4500 r.p.m. for 15 min, and the supernatant was harvested. All infected sheep in the present experiment were inoculated with this virus pool.

Experimental animals

Eight Finnish and 3 Texel sheep served as experimental animals. Of these animals, 5 Finnish sheep (4 females and 1 male) were born in the sheep flock of the Veterinary College, Helsinki, while 3 Finnish and 3 Texel sheep (females) were born on a Finnish sheep farm and were taken to the Veterinary College 2 months before the start of the experiment. Six of the 11 sheep were pregnant at the time of inoculation. In the experiment, the sheep were kept in an isolation unit, where they were free to move and were in close contact with each other. They were fed on hay supplemented with pelleted grain, vitamins and minerals. The sheep were inoculated at the age of 9 to 10 months.

Inoculation of sheep

Three Finnish sheep (Nos. 10, 11 and 1008) and 1 Texel sheep (No. 1320) were inoculated in Rompun (Bayer) analgesia intrapulmonarily with 10 ml, intranasally with 2 ml and intratracheally with 2 ml of maedi M88 strain per sheep. Thus each sheep received approx. 7×10^6 TCID50 of strain M88.

Two Finnish sheep (Nos. 1068 and 1360) and 2 Texel sheep (Nos. 1240 and 1296) were inoculated in Rompun analgesia and with local anesthesia intracerebrally with 1 ml of strain M88. Thus each animal received approx. 5×10^5 TCID50 of strain M88. The inoculum was injected into the deep of thalamus and along

the needle tract, as the needle (of size 0.80×38 mm) was withdrawn (*Sigurdsson et al.* 1957).

A Finnish male sheep (No. 14) was hyperimmunized for antibody production and subcutaneously (s.c.) inoculated with 5×10^7 TCID50 of the M88 strain emulsified in Freund's complete adjuvant (Difco Laboratories). The inoculation was repeated at 2-week intervals thereafter, altogether 4 times, intravenously (i.v.) with 5×10^7 TCID50 of maedi virus.

Two Finnish sheep (Nos. 15 and 16) served as uninoculated control animals and were in close contact with the inoculated ones.

Sacrifice

The sheep were killed by administration of Nembutal (Abbot) i.v. Tissues for virus isolation were aseptically removed, and specimens were obtained for histology. Some of them were frozen at —70°C.

Virus isolation

Heparinized blood. Initially, during 2 to 8 months after inoculation, the mononuclear leukocytes were isolated by centrifugation from 10 ml of heparinized blood through 7 ml of Ficoll-Paque (Pharmacia, Uppsala), and not less than 10^6 viable mononuclear cells were used for virus isolation. Later, leukocytes were isolated from the buffy coat of heparinized blood, and red cells were hemolyzed by adding NH_4Cl-tris buffer solution, and at least 5×10^6 viable leukocytes were used. The cells were washed twice in Hank's balanced salt solution (HBSS, Orion) and diluted in MEM plus 2 % LS. The suspension was added to prewashed SCP cells.

The cultures were observed for at least 2 weeks. If no cytopathogenic effect (CPE) was seen in 2 weeks, the cells were scraped off and passed and observed for at least another 2 weeks. If still no CPE was seen, some of the cultures were passed 1 further time and observed for 2 weeks.

Explant cultures. Samples of each tissue were minced into small fragments, washed several times with HBSS and explanted in plastic 25 cm^2 flasks. The growth medium was MEM plus 20 % LS. When confluent outgrowth of cells had formed, the growth medium was replaced by maintenance medium MEM plus 2 % LS, and the cells were observed during 2—4 weeks at

least, part of them 6—8 weeks; if no changes appeared, the cells were scraped off into medium and passed into SCP cultures. These cultures were again observed during 3—4 weeks at least and some of them were passed once more if there were no changes.

Inoculation of cell culture. Pieces from each tissue were suspended in MEM plus 2 % LS to make a 10 % suspension. Prewashed SCP cells were ionculated with the suspensions and incubated 1 day at 37°C, followed by washing once with HBSS and refeeding with MEM plus 2 % LS.

These cultures were observed for 2 weeks at least; if no changes were seen, they were then passed into fresh SCP cultures and observed another 3—4 weeks at least; even then if no changes were seen, some of the samples were passed once more.

Virus titration

SCP cells on microplates (Flow, U-shaped wells) were prewashed before inoculation. Virus dilutions were prepared in MEM plus 2 % LS. Four wells were inoculated with 0.2 ml of each 10-fold dilution. The culture plates were maintained in a humified CO_2 incubator and read for CPE. Viral titers were determined from 50 % end points (*Reed et al.* 1938).

RESULTS

Virus isolation

The experimental exposure of SCP-passed maedi M88 strain resulted in infections in the intracerebrally as well as in the intrapulmonarily inoculated sheep. Virus has been isolated from peripheral blood leukocytes of all experimentally infected sheep. Sequential virus isolations starting 2 months after inoculation are presented in Table 1. Maedi virus was quite regularly isolated from the intrapulmonarily inoculated sheep, beginning 2—3 months after inoculation, 1 sheep excepted in which viremia could not be demonstrated until 6 months after inoculation. No virus could be isolated from the intracerebrally inoculated sheep during the first 7 months after inoculation. At 8 to 11 months, maedi virus began to be detectable in the group of the intracerebrally inoculated sheep. In the case of the uninoculated control sheep no maedi virus was recovered from peripheral blood leukocytes during 14 months after inoculation. Maedi virus could

Table 1. Virus isolation from the peripheral leukocytes of the experimental sheep after inoculation of maedi M88 virus.

Time in months after inoculation	Sheep inoculated intrapulmonarily				intracerebrally				Uninoculated		s.c. and i.v.
	10	11	1008	1320	1068	1240	1296	1360	15	16	14
0	—	—	—	—	—	—	—	—	—	—	—
2	+	—	+	—	—	—	—	—	—	—	—
3	+	+	+	—	—	—	—	—	—	—	—
4	+	+	+	—	—	—	—	—	—	—	—
5	+	+	+	—	—	—	—	—	—	—	+
6	+	+	+	+	—	—	—	—	—	—	—
7	+	+	+	+	—	—	—	—	—	—	—
8	+	+	+	+*	—	+	—	—	—	—	+
9	+	+	+		—	+	+	—	—	—	—
10	+	+	+		+	—	+	—	—	—	+
11	+	+	+		—	+*	+	+	—	—	+
12	+	+	—		+		+	+	—	—	+
13	+	—	+		+		+	+	—	—	—
14	—	—	+		—		+	—	—	—	—
15	+	+	—		—		+	+	—	+	+
16	—	+	—		—		—	—	—	—	—

* = time of death.
— = negative virus isolation.
\+ = positive virus isolation.

be isolated from 1 uninoculated control sheep 15 months after inoculation of the other sheep.

Maedi virus was irregularly isolated from the Finnish male sheep No. 14, which was given s.c. and i.v. injections of maedi M88 virus.

Clinical signs

Distinct symptoms of a clinical disease were seen in 1 intrapulmonarily inoculated sheep, about 8 months after inoculation. The Texel sheep No. 1320 developed clinical signs with an appearance suggesting combined maedi and visna. This animal first had a dry cough and dyspnea during 3 months. Eight months after inoculation, the animal also had a gastrointestinal disorder of a few days' duration; the animal also rested the distal end of metatarsus. The gastrointestinal disorder disappeared, but paraplegia developed in a couple of days. The sheep was still alert and had a good appetite, merely its respiration was labou-

red and it had a dry cough; the urine was strongly yellow. The animal was sacrified after 4 days of manifest paraplegia, i.e. 8½ months after inoculation.

The Texel sheep No. 1240 died suddenly, 11 months after intracerebral inoculation of maedi M88 strain, without observed clinical signs.

The rest of the experimental sheep displayed no clinical signs of maedi or visna during the observation period of 18 months.

Virus isolation after necropsy

After necropsy, maedi virus was recovered from the intrapulmonarily inoculated sheep No. 1320 as well as from the intracerebrally inoculated sheep No. 1240 (Table 2). Maedi virus was isolated from lungs, spleen and mediastinal lymph node of sheep No. 1320 and from the central nervous system of sheep No. 1240.

T a b l e 2. Virus isolations from 2 experimental sheep after necropsy.

Origin of tissue sample	1320	1240
Choroid plexus	—	ND
Cerebellum	—	+
Medulla	—	+
Cerebrum	—	+
Spleen	+	—
Lungs	+	—
Mediastinal lymph node	+	—

ND = not done.
\+ = positive virus isolation.
— = negative virus isolation.

Pathology

At the necropsy of sheep No. 1320, its lungs were found macroscopically to have increased in weight and size, with a spongy appearance; the liver was yellow and the muscles of the forelimbs appeared degenerated. Histopathological signs of maedi were observed in the lungs, with chronic progressive interstitial inflammation and lymphoid proliferation. No histopathological changes of visna were seen in the central nervous system. The histopathological examination confirmed jaundice and muscle degeneration.

The necropsy of the intracerebrally inoculated sheep No. 1240 revealed no macroscopical changes elsewhere but in the liver, which was bloody and enlarged. Histopathological examination revealed chronic progressive interstitial pneumonia. No histopathological changes compatible with visna were observed in the central nervous system. Histopathological examination also revealed nephrosis and widespread necrosis in the liver. This hepatic necrosis is a possible cause for the animal's sudden death. The necrosis might be due to thrombi or to a parasite, but no evidence of parasite was found. The spleen of sheep No. 1240 was very small and atrophic.

DISCUSSION

The maedi strain M88 was no recent isolate: it was isolated from maedi-affected lungs in 1961 in Iceland and had been passed many times (exact number not known) in SCP cells and had been kept at —70°C many years since isolation. It is therefore conceivable that the strain has lost some of its virulence for sheep owing to the cell culture passages. The strain was passed 4 times in cell culture in Finland before being used for animal inoculations.

Experimental inoculation of the sheep with Icelandic maedi strain M88 regularly resulted in infection. Viremia could be demonstrated in all inoculated sheep in accordance with the results by *De Boer* (1975) with zwoegerziekte virus and those of *Nathanson et al.* (1976) with visna virus.

There was a striking difference in the demonstration of viremia after inoculation between the groups of intrapulmonarily and intracerebrally inoculated sheep, as shown in Table 1. In the first group, viremia could be demonstrated in all animals within 2—6 months, and in the latter group in none within 7 months after inoculation. An explanation why viremia was demonstrated clearly later in the intracerebrally inoculated sheep could be that the M88 strain is not highly neurotropic. The M88 strain might replicate very slowly in the brains and might escape to the periphery of the body only late, while M88 replicated well in the lungs of the intrapulmonarily inoculated sheep, resulting in earlier viremia. Another possible explanation is the difference in virus quantity. The intrapulmonarily inoculated sheep received 14 times more of the M88 strain than the intracerebrally inoculated sheep. How, if at all, this might result in a difference in

replication speed is not clear. In our opinion the low neurotropism of the maedi M88 strain would appear more likely as explanation for the phenomenon observed. It remains to be explained whether the postulated lower neurotropism could be explained by a higher tendency of the virus to exist in the provirus state in brain cells compared with the cells of the respiratory tract. The results of *Haase et al.* (1977) regarding the occurrence of provirus of visna in SCP tissues could support this hypothesis.

If few infected leukocytes occurred in the blood, the demonstration of viremia would not be easy. *Petursson et al.* (1976) pointed out that only very few cells (less than 1 per 100 000) of buffy coat of chronically infected visna sheep yield virus. In the course of our studies we decided to increase the number of leukocytes to be used in virus isolation, hoping that we could thus increase the sensitivity in demonstration of viremia. During 2—8 months after inoculation at least 10^6 mononuclear leukocytes isolated by Ficoll-paque gradient were used. After 8 months, no less than 5×10^6 leukocytes from the buffy coat were used for virus isolation. Mononuclear cells, lymphocytes in particular, seem to serve as a site of virus replication (*Petursson et al.*). After we increased the number of leukocytes, viremia could be demonstrated in the Texel sheep No. 1296 at 9 months, in the Finnish sheep No. 1068 at 10 months and in the Finnish sheep No. 1360 at 11 months after intracerebral inoculation of maedi virus. Viremia was already demonstrated in the Texel sheep No. 1240, 8 months after inoculation. After the first demonstration of viremia, maedi virus was irregularly recovered in these sheep. Further discussion of the phenomenon of difference in types of infection between the groups, as regards immune response, will follow in Part 2 of this work (*Sihvonen et al.* 1980).

Maedi diagnosis was confirmed by virus isolation and histopathological examination in the intrapulmonarily inoculated Texel sheep No. 1320 showing clinical signs suggesting combined maedi and visna. No histopathological changes of visna were found, nor was any virus isolated from the central nervous system or from the cord, an altogether unexplained surprise. Cases with combined maedi and visna signs occur frequently in experimental inoculations and sometimes also in infected sheep flocks (*Gudnadottir* 1974), and gradually progressing paraplegia or even total paralysis are typical clinical signs of visna. The

muscle degeneration which was found could be nutritional myopathy, but the thought is quite remote that such changes could account for paraplegia. Of course, though just as improbably, the sheep might have been paralysed owing to an affection of the liver. The cause of paraplegia could not be confirmed.

The intracerebrally inoculated Texel sheep No. 1240 displayed no histopathological lesions compatible with visna, but maedi virus was isolated from its central nervous system. This sheep had developed pulmonary lesions of maedi. This finding supports the hypothesis that the maedi M88 strain used in these experiments is more strongly pulmopathogenic than neuropathogenic.

REFERENCES

De Boer, G. F.: Zwoegerziekte virus, the causative agent for progressive interstitial pneumonia (maedi) and meningo-leucoencephalitis (visna) in sheep. Res. Vet. Sci. 1975, *18*, 15—25.

Gudnadottir, M.: Visna-maedi in sheep. Progr. med. Virol. 1974, *18*, 336—349.

Gudnadottir, M. & P. A. Pálsson: Transmission of maedi by inoculation of a virus grown in tissue culture from maedi-affected lungs. J. infect. Dis. 1967, *117*, 1—6.

Haase, A. T.: The slow infection caused by visna virus. Curr. Top. Microbiol. Immunol. 1975, *72*, 101—156.

Haase, A. T., L. Stowring, O. Narayan, G. Griffin & D. D. Price: Slow persistent infection caused by visna virus: Role of host restriction. Science 1977, *195*, 175—177.

Harter, D. H., R. Axel, A. Burny, S. Gulati, J. Schlom & S. Spiegelman: The relationship of visna, maedi and RNA tumor viruses as studied by molecular hybridization. Virology 1973, *52*, 287—291.

Mårtensson, J.-E. & G. Holm: Maedipneumoni hos får i Sverige. (Sheep maedi pneumonia in Sweden). Svensk Vet.-Tidn. 1975, *27*, 283—284.

Nathansson, N., H. Panitch & G. Petursson: Pathogenesis of visna: Review and speculation. *In* Kimberlin, R. H. (ed.): Slow Virus Diseases of Animals and Man, Frontiers in Biology. Vol. 44. North Holland Publishing Co., Amsterdam and Oxford 1976, 115—131.

Pálsson, P. A.: Maedi and visna in sheep. *In* Kimberlin, R. H. (ed.): Slow Virus Diseases of Animals and Man, Frontiers in Biology. Vol. 44. North Holland Publishing Co., Amsterdam and Oxford 1976, 17—43.

Petursson, G., N. Nathansson, G. Georgsson, H. Panitch & P. A. Pálsson: Pathogenesis of visna. 1. Sequential virologic, serologic and pathologic studies. Lab. Invest. 1976, *35*, 402—412.

Reed, L. J. & H. S. Muench: Simple method estimating fifty per cent end points. Amer. J. Hyg. 1938, *27*, 493—497.

Sigurdsson, B., P. A. Pálsson & H. Grimsson: Visna a demyelinating transmissible disease of sheep. J. Neuropath. exp. Neurol. 1957, *16*, 389—403.

Sihvonen, L., T. Estola & J. Tuomi: Experimental maedi infection in sheep. 2. Antibody response. Acta vet. scand. 1980, *21*, 124—133.

Thormar, H. & B. Sigurdardottir: Growth of visna virus in primary tissue cultures from various animal species. Acta path. microbiol. scand. 1962, *55*, 180—186.

SAMMANFATTNING

Experimentell maedi infektion hos får. 1. Upptäckt av virus, klinisk bild, histopatologi.

Åtta får inokulerades med en isländsk maedi M88 stam, två oinokulerade får fungerade som kontroller och var i nära kontakt med de inokulerade. Hälften av fåren inokulerades via luftvägarna med 7×10^6 TCID50 av M88 stammen och den andra hälften intracerebralt med 5×10^5 TCID50 av den samma stammen.

Maedi M88 stammen har isolerats från perifera blodleukocyter av alla inokulerade får. Skillnaden mellan de två grupperna beträffande påvisad viremi efter inokulationen var slående. Viremi demonstrerades i de intrapulmonalt inokulerade fåren inom 2—6 månader men i de intracerebralt inokulerade fåren först 8—11 månader efter inokulationen. Denna upptäckt reflekterar högst antagligen den använda stammens svaga neurotropism. Efter viremins första påvisande (till 16 månader efter inokulationen) har maediviruset åter isolerats ganska regelbundet i perifera blodleukocyter hos alla de intrapulmonalt inokulerade får och mindre regelbundet hos de intracerebralt inokulerade. Maedi virus isolerades från en av de oinokulerade kontrollfåren 15 månader efter inokulationen.

Det första kliniska fallet som såg ut som ett förenat falla av både maedi och visna hittades bland de intrapulmonalt inokulerade fåren 8½ månad efter inokulationen. Den histopatologiska undersökningen och virusisoleringen bekräftade maedi. Orsaken till paraplegin kunde inte bekräftas. Inga histopatologiska förändringar kunde påvisas och ingen virusisolering gjordes från detta fårs centralnervösa system.

Ett av de intracerebralt inokulerade fåren dog plötsligt utan att några kliniska tecken observerades elva månader efter inokulationen. Den histopatologiska undersökningen avslöjade lungförändringar typiska för maedi men inga visnaförändringar i dess centralnervösa system. Däremot isolerades maedi virus från olika delar av hjärnan.

Under en 18 månaders observationsperiod upptäckes inga kliniska symptom som tydde på maedi eller visna hos de andra försöksfåren.

(Received November 12, 1979).

Reprints may be requested from: Liisa Sihvonen, the Department of Microbiology and Epizootology, College of Veterinary Medicine, Box 6, 00551 Helsinki 55, Finland.