Isolation of a Viral Agent from the Lungs of Sheep Affected with Maedi

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Maedi is a disease of sheep characterized by slow, progressive, fatal pneumonia (Gíslason, 1947). The main pathologic features are greatly increased lung weight with changes in color and consistency and proliferation of the mesenchymal tissue of the lungs (Sigurdsson et al, 1952; Gíslason, 1963). In the years 1945 to 1952 transmission experiments were carried out which indicated that maedi is a virus infection, probably with an incubation period of 2 to 3 years (Sigurdsson et al, 1953).

The disease was prevalent in Iceland from 1939 to 1952 and caused heavy losses in the sheep stock. In 1944 to 1952 the entire sheep stock in the affected districts was slaughtered in an attempt to eradicate the disease. However, since 1952 maedi has reappeared in small epidemics in the western and northwestern part of Iceland.

In the fall of 1958 cases of maedi were found in the western part of the country. Attempts were made to isolate virus from sheep lungs which showed characteristic maedi lesions by using the method which had previously been successfully employed for the propagation of visna virus in tissue culture (Sigurdsson et al, 1960). A viral agent was isolated from 7 out of 8 lungs (B. Sigurdsson, personal communication).

In the spring of 1959 the disease was observed in another area in the northwestern part of Iceland. In the fall of that year the sheep flocks in the area were slaughtered, the lungs were brought to the laboratory, and attempts were made to isolate virus from some of them.

In the present communication the results of these attempts will be reported. Some properties of the isolated agent will be described and its possible relationship with maedi will be discussed.

METHODS

Tissue culture.—Serially propagated cell cultures derived from the chorioid plexus of sheep brain (Sigurdsson et al, 1960) were used for some of the primary isolations, for passing and titrating the virus, and for the neutralization tests. The cultures were grown in medium 199 (Glaxo) enriched with 20% sheep serum. The medium contained 100 units of penicillin and 100 μg of streptomycin per ml. This type of culture will be referred to as a plexus culture. Figure 1 shows an uninfected plexus culture.

Isolation and passage of virus.—For isolation experiments pieces were cut from the dorsal part of the lungs and prepared by one of 2 methods denoted as the explantation and the inoculation methods, respectively.

In the explantation method, pieces of lung tissue were minced with scissors, washed several times with medium 199, and explanted in a clot of chicken plasma. Nutrient medium consisting of medium 199 with 20% sheep serum and antibiotics as above was added, and the
cultures were incubated in a roller drum at 37°C. After 1 to 2 weeks, when a confluent outgrowth of cells had formed, the nutrient fluid was changed to medium 199 with 2% sheep serum (maintenance medium). During the incubation period the maintenance medium was changed every 3 to 4 days. When a cytopathic effect (CPE) commenced to develop in the outgrowths from the lung explants, 0.2 ml of undiluted culture fluid were added to tubes of fresh plexus culture. In later passages the same amount of either undiluted fluid or tenfold dilutions of it was passed into plexus culture tubes.

**Figure 1.**—A monolayer of plexus cells stained with Giemsa. 140X.

**Figure 2.**—Plexus culture infected with a strain of the agent isolated from maedi lungs. Multinucleated cells and stellate cells can be seen. Giemsa stain. 140X.
In the inoculation method, pieces from maedi lungs which had occasionally been stored at $-50\, ^\circ\text{C}$ for some time were suspended in Hanks' salt solution to make a $10\%$ suspension. This was centrifuged at 2500 rpm for 10 minutes, and 0.2 ml of the supernatant fluid were inoculated into each of 10 plexus culture tubes. The inoculated cultures were incubated in a roller drum at 37 $^\circ\text{C}$, and the medium was changed on the following day. They were incubated for a further 18 to 38 days. When CPE was observed in the cell layer, the culture fluid was passed on to new plexus cultures as described for lung explants.

**Virus titrations.**—Serial tenfold dilutions of infected fluid were inoculated in 0.1-ml amounts into each of 3 culture tubes containing 1 ml of maintenance medium. The tubes were examined for CPE every 3 to 4 days for 3 weeks. The $50\%$ end point was calculated by the method of Reed and Muench (1938) and is expressed as TCID$_{50}$ per 0.1 ml.

**Neutralization tests.**—Serial twofold dilutions of serum were mixed with a virus dilution containing about 100 infective doses per 0.1 ml. The mixtures were held at 0 $^\circ\text{C}$ for 48 hours and then added to culture tubes as described by Sigurdsson et al (1960). Simultaneous titration of the virus was included in all neutralization experiments. The culture tubes were placed in a roller drum at 37 $^\circ\text{C}$, and final readings were made 21 days after inoculation.

**RESULTS**

Lungs from the maedi-infected flock may be divided into 2 groups: (1) lungs showing macroscopic changes due to maedi, and (2) lungs without detectable maedi changes. A control group consisted of 12 lungs from healthy sheep of the same age group as the infected animals (2 to 10 years old) and coming from an area in the southwestern part of the country where maedi was eradicated several years ago. Table 1 shows the number of successful isolations of virus from each group of lungs.

In the first group more than 24 hours had usually elapsed from the time of slaughtering until the lungs were received at the laboratory, but in spite of this some of the lungs were explanted. From these lungs the cell growth was rather slow; but a confluent outgrowth was formed, and CPE appeared 20 to 23 days after explantation. A few of the lungs arrived in a fresh condition about 2 hours after slaughtering. The cell growth from these lungs was more abundant, and CPE was sometimes observed as soon as 10 to 14 days after explantation.

Most of the lungs in the second group were also brought to the laboratory in a fresh condition about 2 hours after slaughtering. They were explanted and grew abundantly in the culture tubes. In 2 lungs, weighing 730 g and 620 g, CPE developed in the primary explants, while in cultures from the 2 lightest lungs there was no visible sign of CPE 30 days after explantation, the CPE not becoming detectable until the first passage.

Multiplication of the cytopathogenic agent in the tissue cultures was demonstrated by serial limiting dilution passages, a final dilution of $10^{-20.7}$ of the fluid from the original explant still producing CPE. Most of the strains of the agent were passed 10 to 17 times. In the last passages undiluted fluid produced

| Table 1.—Isolation of a viral agent from lungs derived from a flock of sheep affected with maedi |
|-----------------|-----------------|-----------------|-----------------|
| **Group**      | **Number of lungs** | **Average weight of lungs, g** | **Ratio of successful virus isolations** |
| 1*             | 12              | 1150            | 12/12           |
| 2              | 5               | 695             | 4/5             |
| 3              | 12              | 500             | 0/12            |

* Groups 1 and 2 were from maedi-affected flocks. Group 3 represents controls of lungs from a healthy flock.
maximum CPE in approximately 7 days, with the infectivity titer rising to $10^{6.8}$.

The only lung in the second group which did not yield the agent was from a ram and was not fresh when received at the laboratory. This lung was heavily infected with worms, which might account for the lung weight of about 1100 g. The inoculation method was used, but the primary culture and 3 serial blind passages were all negative.

The lungs from the maedi-free area were explanted in very fresh condition about half an hour from the time of slaughtering. The cell growth became luxuriant in a few days, and the cultures were maintained for 30 days before the first passage. Five serial passages of cells and fluid were carried out at intervals of about 30 days without any sign of CPE.

Cultivation of the agent in other types of tissue culture.—The tissue culture-adapted strains of the agent were successfully propagated in cell cultures which had been prepared from normal lamb lungs by collagenase treatment (Hinz et al., 1959) and which looked much like plexus cultures, being apparently fibroblastic. The agent was also grown in cultures of sheep kidney cells. In both types of culture it reached a titer comparable to that obtained in plexus culture.

Cytopathic changes.—The first visible CPE in cell cultures infected with the agent was increased refractility of the cells, usually beginning at the edge of the cell layer and spreading from there over the whole culture. Later, stellated giant cells were observed and, still later, the cell sheet became discontinuous due to dislodgement of destroyed cells.

In cultures of explanted maedi lungs which were fixed and Giemsa-stained at the time of first passage, only a few of the cells were affected. Cells with pathologic changes and cells in mitosis were observed side by side. Some of the affected cells appeared to have fused together to form irregularly shaped syncytiata. Others were spindle-shaped with 2 to 3 nuclei, a small amount of darkly staining cytoplasm, and long processes extending from the cell body. In fixed and stained plexus cultures inoculated with subpassages of the agent multinucleated giant cells were seen to contain as many as 40 nuclei, either forming a cluster or arranged in a rosette-like pattern (figure 2). Increasing the serum concentration in the maintenance medium seemed to cause an increase in the size and number of nuclei in the giant cells.

Inclusion bodies have not been detected in hematoxylin and eosin-stained or in Giemsa-stained infected cultures.

Neutralization tests.—Preliminary experiments were carried out to see whether sera from sheep affected with maedi would neutralize the newly isolated agent. The strain employed was K34 isolated in the fall of 1958. The sera tested were collected in the fall of 1959 from the maedi-affected sheep flock. The results listed in table 2 show that all the sera derived from sheep with typical signs of maedi neutralized the agent, most of them, however, in very low titer. Only half of the sera from sheep with no

<table>
<thead>
<tr>
<th>Group</th>
<th>Average lung weight, g</th>
<th>Number of positive sera in each dilution</th>
<th>Number of negative sera</th>
<th>Ratio of positive sera</th>
</tr>
</thead>
<tbody>
<tr>
<td>1*</td>
<td>1150</td>
<td>7</td>
<td>1</td>
<td>17/17</td>
</tr>
<tr>
<td>2</td>
<td>560</td>
<td>4</td>
<td>0</td>
<td>4/8</td>
</tr>
</tbody>
</table>

* Group 1 represents sheep with lungs showing macroscopic changes of maedi, while the lungs of sheep in group 2 were free of detectable maedi changes.
signs of maedi neutralized the agent, and these were only effective at the highest concentration tested.

Neutralization tests were also carried out to compare some of the isolated strains. Three sera (nos. 5520, 5511 and 5524) which had been found to neutralize strain K34 at a dilution of 1:16 (table 2) were tested against 10 strains isolated in the fall of 1959. The results are shown in table 3. The sera showed very similar titers against all the strains.

Other properties of the agent.—The agent passed through a gradocol membrane with an average pore diameter of 530 mμ. Preliminary electron microscopic studies of tissue cultures infected with the agent revealed spherical virus-like particles approximately 80 mμ in diameter.

The agent retained its infectivity for at least several months at −50°C, and at −20°C also, although not as well. It could withstand a few cycles of freezing and thawing.

In medium 199 at pH 8 and 37°C the infectivity was gradually lost in 48 hours or less, as shown in table 4.

The infectivity was lost by 24-hour treatment with diethyl ether at 4°C.

DISCUSSION

Previous observations on maedi (Sigurdsson et al, 1952; G. Gislason, personal communication) as well as transmission experiments (Sigurdsson et al, 1953) indicated that the disease was contagious and suggested that it might be caused by a virus. Therefore, when a new epidemic of maedi appeared in 1958, attempts were made to isolate virus from the lungs of typical maedi cases by using a tissue culture method which had previously proved successful in the isolation of the agent of visna, another slowly developing infection of sheep (Sigurdsson et al, 1960). These attempts led to the isolation of 7 strains of a virus-like agent in the fall of 1958 (B. Sigurdsson, personal communication) and of 16 additional strains in the fall of 1959. All the isolated strains showed identical growth patterns in tissue culture, characterized by very slow multiplication of the infective agent and concurrent slow development of CPE. They seemed to be antigenically related when subjected to neutralization tests (see table 3). With respect to other characteristics studied, i.e., ether sensitivity and appearance in the electron microscope, the strains seemed to be identical to one another and similar to visna virus. A study of the relationship between visna virus and the present agent is now in progress.

As to the relationship between the newly isolated agent and maedi, a few facts may be pointed out. Firstly, it was most readily isolated from the lungs which showed the most conspicuous pathologic changes typical of maedi, while attempts to isolate the virus from the lungs of sheep from a maedi-free flock all failed. On the other hand, the agent was isolated from lungs free of macroscopic maedi lesions but deriving from sheep in the maedi-

<table>
<thead>
<tr>
<th>Strain</th>
<th>Serum 5520</th>
<th>Serum 5511</th>
<th>Serum 5224</th>
</tr>
</thead>
<tbody>
<tr>
<td>72</td>
<td>1:32*</td>
<td>1:16*</td>
<td>1:16</td>
</tr>
<tr>
<td>Snilld</td>
<td>Not tested</td>
<td>1:16</td>
<td>Not tested</td>
</tr>
<tr>
<td>108Bf</td>
<td>Not tested</td>
<td>1:8</td>
<td>1:32</td>
</tr>
<tr>
<td>0786</td>
<td>Not tested</td>
<td>1:5</td>
<td>1:32</td>
</tr>
<tr>
<td>Tinna</td>
<td>1:16</td>
<td>1:8</td>
<td>1:16</td>
</tr>
<tr>
<td>62</td>
<td>1:16</td>
<td>1:8</td>
<td>1:16</td>
</tr>
<tr>
<td>Spira</td>
<td>1:16</td>
<td>1:5</td>
<td>1:32</td>
</tr>
<tr>
<td>Frída</td>
<td>1:16</td>
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<tr>
<td>0676</td>
<td>Not tested</td>
<td>1:8</td>
<td>Not tested</td>
</tr>
<tr>
<td>103M</td>
<td>Not tested</td>
<td>1:8</td>
<td>Not tested</td>
</tr>
<tr>
<td>K34</td>
<td>1:16</td>
<td>1:16*</td>
<td>1:16*</td>
</tr>
</tbody>
</table>

* These sera were also tested after preheating at 60°C for 30 minutes and did not show an altered neutralizing titer.

Table 4.—Decrease in infectivity at 37°C

<table>
<thead>
<tr>
<th>Time, hours</th>
<th>0</th>
<th>9</th>
<th>21</th>
<th>35</th>
<th>48</th>
</tr>
</thead>
<tbody>
<tr>
<td>Virus titer</td>
<td>$10^{5.3}$</td>
<td>$10^{4.2}$</td>
<td>$10^{2.2}$</td>
<td>$10^{1.2}$</td>
<td>$10^{2}$</td>
</tr>
</tbody>
</table>

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affected flock (group 2, table 1). In explants of these lungs CPE developed much less readily than in explants of lungs from typical maedi cases, indicating a lower content of the cytopathogenic agent.

Sigurdsson et al (1953) noticed scattered microscopic lesions in the lungs of experimental sheep that were infected with maedi but did not yet show macroscopic changes except for a slight, but significant, increase in lung weight. They interpreted the microscopic lesions as being due to a beginning maedi infection. Furthermore, epidemiological studies of maedi have shown (G. Gislason, personal communication) that during the first 2 years after maedi is introduced into a susceptible flock of sheep the lungs are very rarely found, although they may be detected microscopically. In our study a histological examination was not carried out, but the lungs of group 2 showed somewhat increased weight as compared with the control lungs (table 1). This might suggest that they were in an early stage of maedi infection and therefore contained a lower concentration of virus than was found in the lungs with macroscopic maedi lesions.

Sera from clinical cases of maedi neutralized the agent, while sera from apparently healthy sheep from the same flock were either negative or showed neutralization only at high serum concentration (table 2). A virus strain (K34) isolated in the fall of 1958 from a maedi sheep in the western part of the country was neutralized by sera collected in a distant district a year later from another flock of sheep affected with maedi. Sera collected in districts free of maedi have so far not been found to neutralize the virus.

The above findings suggest an etiological relationship between the newly isolated virus-like agent and the slow progressive pneumonia of sheep known as maedi.

**SUMMARY**

Sixteen strains of a cytopathogenic agent with the properties of a virus have been isolated from the lungs of 17 sheep from a maedi-affected flock. Attempts to isolate virus from the lungs of 12 sheep from a maedi-free flock failed. The virus was more readily isolated from lungs showing macroscopic lesions of maedi than from apparently healthy lungs from the same flock. The virus grew very slowly in tissue culture and produced a cytopathic effect characterized by the formation of multinucleate giant cells. Serum from sheep affected with maedi neutralized the virus.

**REFERENCES**


