Electron microscope studies have indicated that visna and mædi viruses are formed by budding at the surface of their host cells (Thorner 1961, Thorner 1965), but did not reveal any specific structural changes, either in the nuclei or in the cytoplasm. Observations of visna virus-infected cell cultures stained with acridine orange suggested an increase in the amount of single-stranded RNA in the cytoplasm of infected cells (Thorner 1966). The cytoplasm fluoresced uniformly orange-red without much particulate staining. The orange-red fluorescence was remarkably intense in the cytoplasmic processes of stellate cells and at the surface of degenerating cells. No specific changes were observed in the cell nuclei.

The present study was undertaken in an attempt to learn more about host cell-virus interactions in cell cultures infected with visna and mædi viruses, particularly with regard to intracellular formation of virus antigen during the growth cycle.

**MATERIALS AND METHODS**

*Cell cultures.* Serial cultures of sheep choroid plexus cells (Sigurdsson, Thorner & Pálsson 1960) were grown in medium 199 containing 20 per cent sheep serum and maintained in medium 199 with 2 per cent sheep serum. Monolayer cultures grown on 11 × 22 mm coverglasses in Leighton tubes were used in all experiments.

*Infection of cultures with virus.* Visna virus, strain K796, and mædi virus, strain M88, were used (Thorner 1965, Thorner & Helgadóttir 1965). Each coverslip culture was infected with virus at an input multiplicity of about 10 TCID<sub>50</sub> per cell and rotated for 3 to 4 hours in a roller drum at 37°C. The cell layers were then washed 3 times with medium 199 and 1.5 ml of maintenance medium added to each Leighton tube. The tubes were rotated again at 37°C until harvested for fixation and staining of the cell layer and for titration of virus in the fluid medium (Thorner & Helgadóttir 1965).

*Antiserum.* A number of sheep antisera against visna and mædi viruses were collected from sheep at intervals varying from 5 weeks to 49 months after intracerebral or intrapulmonary inoculation with infective virus. Sera from the same animals collected before inoculation with virus and sera from normal uninfected sheep were

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used as controls. The neutralization titres of the sera were determined as previously described (Thomson 1963, Thomson & Helgadottir 1965). The complement fixation titres were determined by the method of Guadnadal & Kristinadottir (1967). Serum globulins were prepared by precipitation with half-saturated ammonium sulphate and were conjugated with fluorescein isothiocyanate according to the method of Marshall, Eveland & Smith (1958). Fluorescein-labelled globulins were absorbed 3 times with sheep liver powder and once with a suspension of fresh choroid plexus cells in order to eliminate as much as possible of non-specific fluorescent staining.

**Fixation and fluorescent staining of cell cultures.** Washed cell layers were fixed for 10 minutes in dry acetone and stained by the direct fluorescent antibody technique (Coons et al. 1942, Coons & Kaplan 1950) at 37°C for 45 minutes. After thorough washing with phosphate buffered saline and mounting of the coverslips in buffered glycerol, the cells were examined in a Leitz Ortholux fluorescence microscope with a darkfield condenser, using primary filter BG12 and a blue light absorbing barrier filter. Kodak Ektachrome high-speed film was used for colour photography.

**Specificity of staining.** The following criteria were used as proof of specificity of the fluorescent staining (Coons & Kaplan 1950, Cherry, Goldman & Carki 1959): 1) Conjugated antisera stained cultures infected with visna and medl viruses, but not uninfected cultures or cultures infected with vaccinia virus, herpes simplex virus or Newcastle disease virus. 2) Cultures infected with visna or medl viruses were not stained by conjugated normal sera. 3) Staining of infected cells was inhibited by preincubation of the cell layers with unconjugated visna or medl antisera, but not by preincubation with unconjugated control sera. Antiseras against medl virus blocked staining by fluorescein-labelled visna antisera, and vice versa.

**RESULTS**

**Localization of virus antigen in cells harvested at successive stages of infection.** A series of coverslip cultures was harvested at intervals after inoculation with virus. The cell layers were fixed for immunofluorescent staining and samples were removed from the fluid medium for virus titration. Uninoculated control cultures were harvested for fixation and staining at the same intervals as infected cultures.

There was no specific staining in cultures harvested 6 hours after infection with visna virus. A diffuse specific fluorescence was observed in the cytoplasm of a small number of cells in cultures harvested 20 hours post-infection (p.i.), shortly before a rise in visna virus titre was detected in the medium. During the following hours both the number of fluorescing cells and the intensity of fluorescence increased concurrently with the increase in virus titre in the cultures. In some cells the cytoplasmic fluorescence was seemingly most intense around the nuclei, possibly because the thickness of the cytoplasm was greatest in the perinuclear area. At 3 days p.i., about half of the cells showed cytoplasmic fluorescence. It was most intense in stellate cells with a cytopathic effect characteristic for visna virus. In a few cells bright fluorescent staining was observed at the cell surface, apparently in the form of floccules of various sizes (Fig. 1). At 4 days p.i., the floccules had become more pronounced and in some cells the cell periphery showed up as a bright line of varying thickness, being particularly distinct in cytoplasmic processes of stellate cells (Fig. 2). At least some of the antigen was apparently localized outside the cell membrane, since it
Fig. 1.
Three days after infection. Diffuse cytoplasmic fluorescence and bright granules at the cell surface.

Fig. 2.
Four days after infection. Bright fluorescence of the cell membrane.

Fig. 3.
Fluorescent staining without fixation of cells harvested 4 days after infection. Some virus antigen is localized on the outer surface of the cell membrane.

Figs. 1 to 4.
Sheep cells stained with fluorescent antibodies at various times after infection with visna virus. X 750.
was stainable with fluorescent antibodies before fixation (Fig. 3). Cells which were rounding up appeared very bright and their surface was covered with small knobs of brilliant fluorescence (Fig. 4).

Specific fluorescence was not detectable in cultures infected with mädi virus until at least 30 to 40 hours p.i. and it increased slowly during the following days. As in visna, the fluorescence was confined to the cytoplasm and became particularly intense at the cell surface about 6 to 7 days p.i. (Fig. 5). At no time was there evidence of specific nuclear fluorescence in cells infected with visna or mädi viruses.

**Fluorescent antibodies in various visna and mädi antisera.** Several visna and mädi antisera were tested for fluorescent antibodies and an attempt was made to compare the intensity of the fluorescence with the neutralization and complement fixation titres of the sera (Table 1). The fluorescent activities of various sera were compared in infected cell cultures under the same conditions, and were scored as 2 + (bright fluorescence), + (distinct fluorescence), (+) (faint fluorescence), and — (no specific fluorescence). Preinoculation sera and other normal sera
were consistently found to be free of specific fluorescent antibodies against visna and maedi viruses as well as of neutralizing and complement fixing antibodies. A very slight fluorescent activity was found in a serum which was collected 5 weeks after intrapulmonary (i.p.) inoculation with a high tissue culture passage of visna virus (serum no. 5870). This serum had a very low complement fixing activity and no neutralizing activity against the virus. All the other post-inoculation sera were consistently found to contain fluorescent antibodies against the viruses. These sera were collected from sheep after i.p. inoculation with a high tissue culture passage of visna virus (serum no. 6040 and no. 6873), after intracerebral (i.c.) inoculation with a low tissue culture passage of visna virus (serum no. 8752), after i.c. inoculation with a low tissue culture passage of maedi virus (serum no. 14663) and after i.c. inoculation with extract from a visna brain (serum no. 4992). The intensity of the fluorescent staining was found to correlate fairly well with the complement fixation titres of the sera. There was, on the other hand, no correlation between fluorescent staining and the neutralization titres of the sera. Fluorescein-labelled maedi antisera stained the cytoplasm of visna virus-infected cells, and vice versa. However, visna antisera seemed to have somewhat less fluorescent activity against maedi virus than against visna virus.
DISCUSSION

In the present work the localization of visna and maedi antigen in cells harvested at various times after inoculation was studied, using a number of various fluorescein-labelled antisera. A uniform specific fluorescence was observed in the cytoplasm of cells infected with visna and maedi viruses and later it increased to intensity at the cell surface, indicating that virus antigen was formed in the cytoplasm and accumulated in high concentration at the cell membrane. The formation of detectable amounts of viral antigen in infected monolayers coincided with the formation of infectious virus in the cultures. Specific fluorescence could not be detected with certainty in the cell nuclei at any time during the viral growth cycle, regardless of the antiserum used for fluorescent staining. Either there was no viral antigen synthesized in the nuclei of infected cells or such an antigen failed to cause detectable antibody formation in sheep.

The results of the present study are in general agreement with a recently published report by Harter, Hsu & Rose (1967), except that large cytoplasmic inclusions of fluorescent material were not observed at any time during the infection and viral antigen seemed to aggregate only at or outside the cell membrane.

A comparison of the information obtained by electron microscopy and by immunofluorescent and acridine orange staining of cells infected with visna virus indicates that the cytoplasm is the main site of formation of virus material in host cells. Electron microscope observations have shown that, in a late stage of visna and maedi infections when most of the cells in a monolayer have either been turned into stellate forms or are rounding up and detaching from the glass, virus-like particles are found in a large number on the surface of the cells and the cell membranes are covered with small buds, which are believed to be virus particles being formed and released by the cells (Thorner 1961, Thorner 1965). At this stage virus antigen is present in large amounts at the cell surface and partly outside the cell membrane, as shown by immunofluorescent staining of unfixed cell surfaces. At the same time, cytoplasmic processes of stellate cells and the surface of degenerating cells stain brilliantly orange-red by acridine orange (Thorner 1966), indicating high concentration of single-stranded RNA. All these observations indicate that large amounts of viral antigen and RNA are being incorporated into virus particles at the cell membrane. However, the conclusion that visna and maedi virus particles contain RNA is yet to be confirmed by biochemical methods.

Sheep infected with visna and maedi viruses seem to form antibodies detectable by fluorescein labelling shortly after infection and long before neutralizing antibodies are detectable in their sera. It is not known whether the fluorescent antibodies are identical to the complement fix-
ing antibodies which are also formed shortly after infection with visna and maedi viruses.

SUMMARY

The development of virus antigen in sheep cell cultures infected with visna and maedi viruses was studied by th fluorescent antibody technique. A diffuse specific fluorescence was observed in the cytoplasm of infected cells shortly before the formation of new infectious virus. Later the cytoplasmic fluorescence increased in intensity and became particularly brilliant at the cell surface, indicating that virus antigen was formed in the cytoplasm and accumulated in high concentration at the cell membrane, coinciding with maximum virus titre in the cultures. At no time was there evidence of specific nuclear fluorescence in infected cells.

In a number of sheep sera, fluorescent activity was found to correlate with the complement fixation titre, but not with the neutralization titre. Fluorescein-labelled maedi antisera stained cells infected with visna virus, and vice versa.

REFERENCES