Tissue Culture Studies of the Virus of Progressive Pneumonia, A Slow Infectious Disease of Sheep[1-3] (36045)

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(Introduced by R. A. Good)
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Progressive pneumonia is a slowly developing pulmonary disease of sheep. Clinical signs of the disease, mainly loss of weight and increasingly severe respiratory distress, appear 1–4 years after the animals are inoculated with the causative virus (1). At first, the respiratory distress is apparent only after exercise; later, it is evident even when the animal is at rest. The clinical course lasts 3–12 months, and ends fatally. Histologically, the disease is characterized by proliferation of lymphocytes and reticular cells in interalveolar septa of the lungs, which gradually obliterates the air spaces (2).

Diseases of sheep that closely resemble progressive pneumonia clinically and pathologically include *maedi* in Iceland (3), *zwoegerziekte* in Holland (4), Graaf–Reinet disease in South Africa (5), *la bouhite* in France (6), and progressive interstitial pneumonia in Germany (7). Viruses have been isolated and studied from sheep affected with *maedi* and *zwoegerziekte* (8, 9). In 1968, Kennedy *et al.* (10) isolated a virus from the lungs of sheep affected with progressive pneumonia and presented evidence that it is the etiologic agent.

The purpose of this investigation was to study in tissue culture the properties of progressive pneumonia virus (PPV) and to determine its relation to the viruses of *maedi*, *visna*, and *zwoegerziekte*.

Materials and Methods. Cell cultures. Normal sheep lung (NSL) cells were prepared by trypsin dispersion of normal lung tissue (11). Monolayers, grown in Eagle's minimum essential medium containing 10% fetal bovine serum (EMEM), were incubated at 37°. Prepared in this manner, the monolayers consisted of fibroblastic cells that survived about 10 serial passages. Because virus replicated well in only a few cell lines, many cell lines had to be initiated. Forty ampules of first passage of NSL cells were rate-frozen by the method of Perry *et al.* (12). This supply was used throughout this study.

Virus. PPV, no. 64–84, was isolated from a 10% suspension of lung from a sheep affected with experimentally induced disease. The virus had undergone 7–13 serial passages in tissue culture when used in these experiments.

Infection of cell monolayers. Experiments were performed by inoculating confluent NSL cell monolayers, grown on coverslips in Leighton tubes, with 0.2 ml of undiluted virus suspension. After 3 hr adsorption at 37°, the inoculum was removed and the monolayers were washed 3 times with Earle's balanced salt solution (EBSS). EMEM was added, and the tubes were incubated at 37°. At intervals after infection, the medium from 4 tubes was pooled, centrifuged at 1500*g* for 15 min, and stored at −70° until assayed for infective virus. When cell-associated virus

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(CAV) was studied, 2 monolayers were washed with EBSS and the cells were frozen in liquid nitrogen. They were then thawed in warm water, suspended in EBSS by shaking vigorously and centrifuged and stored as described above.

Assay of infective virus. Confluent NSL monolayers were grown in Micro-Test II plates (Falcon Plastics, Oxnard, California). Twelve wells were each inoculated with 0.025 ml of serial 10-fold dilutions of virus in EMEM. Three-tenths ml EMEM was added to each well, the wells were covered with a sheet of pressure-sensitive tape and the plates were incubated in a humidified CO_2 incubator at 37°. After 21 days of incubation, monolayers were washed 3 times with EBSS, fixed with absolute methanol and stained with hematoxylin and eosin. The wells were then filled with water and the stained cells were examined microscopically for polykaryocytes (PKC). Fifty percent tissue-culture infectious dose per ml ($TCID_{50}$ virus/ml) was determined by the Spearman–Karber method (13). Results of this assay correlated well with those obtained by using 4 Leighton tubes per dilution, and saved space, cells, reagents, and time.

Staining procedures. Immunofluorescent studies were performed by the indirect technique (14). Serum used was from a sheep that had been inoculated 54 months earlier with PPV but was still clinically normal. This serum had a complement-fixing antibody titer of 128 and a neutralizing antibody titer of 32, but had to be concentrated 3-fold by pervaporation for optimum fluorescence. Before use, rabbit anti-sheep gamma globulin, conjugated with fluorescein isothiocyanate (Colorado Serum Co., Denver, Colorado) was passed through Sephadex G-25 and absorbed with normal rabbit-kidney powder.

Coverslip preparations were fixed in Bouin's fluid for 15 min and stained with hematoxylin and eosin.

Monolayers on coverslips were stained with acridine orange by the method described by Thormar (15). Digestion with nucleases was performed by the method of Harter *et al.* (16).

Plaque assay. Plaque assays were conducted on NSL cell monolayers in 30 ml plastic flasks by use of Noble agar and carboxymethylcellulose overlays as described by Harter (17).

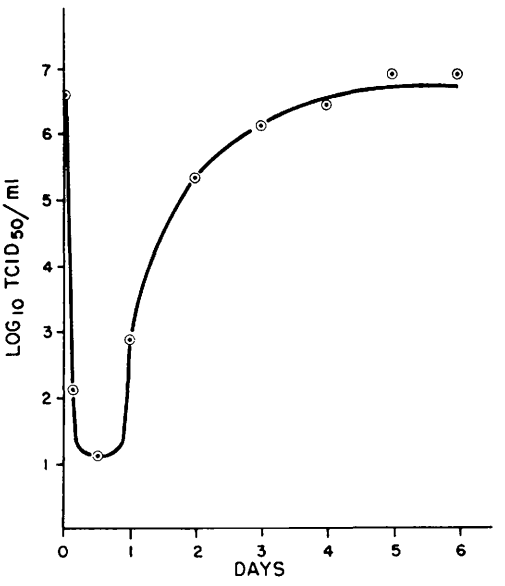

FIG. 1. Growth of progressive pneumonia virus in normal sheep lung cells at 37°. Monolayers were inoculated at a multiplicity of 2 $TCID_{50}$/cell.

Hemagglutination. Chick red blood cells in phosphate buffers of pH 6.0, 6.2, 6.4, 6.6, 6.8 and 7.0 were prepared (18), mixed with tissue-culture fluid containing $10^{6.4}$ $TCID_{50}$ virus/ml and incubated at 4°, 21°, or 37° for 1 hr.

Hemadsorption. Infected NSL cell monolayers were washed 3 times with EBSS 7 days post-inoculation (PI). Each monolayer was covered with 0.2 ml of a 0.4% suspension of guinea pig red blood cells in EBSS and incubated at either 4° or 37° for 30 min.

Results. Growth curves. Replication of PPV in NSL cell monolayers infected with a multiplicity of infection (MOI) of 2 $TCID_{50}$ virus/cell is shown in Fig. 1.

The latent period lasted about 1 day and was followed by 2 days of rapid replication. A plateau of $10^{6.6}$ $TCID_{50}$ virus/ml was reached at 4 days PI. The maximum titer found was $10^{7.3}$ $TCID_{50}$ virus/ml at 5 days PI.

Monolayers were infected with a MOI of 1 $TCID_{50}$ virus/cell to determine the relation-

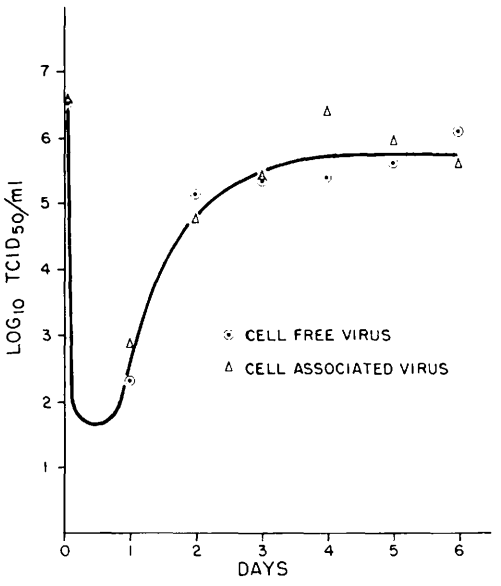

FIG. 2. Comparison of cell-associated and cell-free virus in normal sheep lung cells inoculated with progressive pneumonia virus at a multiplicity of 1 $TCID_{50}$/cell.

ship of cell-associated virus (CAV) to cell-free virus (CFV) (Fig. 2). The latent period for CFV was again 1 day and was followed by 1 day of rapid growth and 4 days of gradual increase. The growth curve for CAV was similar to that for CFV except that high titer of CAV is reached 2 days before the high titer of CFV.

Cytopathic changes in infected monolayers. Coverslips were taken at 3 hr and at 1, 2, 3, 4, 5, and 6 days PI for staining with hematoxylin and eosin, fluorescent antibody, and acridine orange. When a MOI of 2 $TCID_{50}$ virus/cell was used as inoculum, the first PKC were seen at 1 day PI. These involved few cells and contained only 5–10 nuclei. PKC increased in size and number during the next 3 days. By 5 days PI, over 80% of the cells in the monolayer were involved (Fig. 3) and some PKC began to round up and slough from the surface. The PKC contained many nuclei and were darker staining. Cytoplasmic inclusions were not seen.

Specific immunofluorescence was first detected in infected monolayers at 1 day PI. Less than 1% of the cells were involved and the antigen appeared only as small areas in the cytoplasm near the nuclei in PKC and some mononuclear cells. The fluorescence was homogeneous and was more intense near the nuclei. At 2 days PI, many PKC in the monolayer revealed cytoplasmic fluorescence. The intensity of the fluorescence and the number of cells involved increased to a maximum at 5 days PI. About 80% of the PKC were involved and they revealed bright fluorescence around but never in the nuclei (Fig. 4). Fluorescence remained homogeneous and neither cytoplasmic inclusions nor fluorescent particles on the cell surface were observed.

When a MOI greater than 4 $TCID_{50}$ virus/cell was used to inoculate NSL cell monolayers, extensive cell fusion occurred as early as 1 hr PI (Fig. 5). The fusion continued to involve an increasing number of cells until 12 hr PI. After this, most cells involved sloughed from the surface. Viral antigen was not detected in the fused cells of monolayers

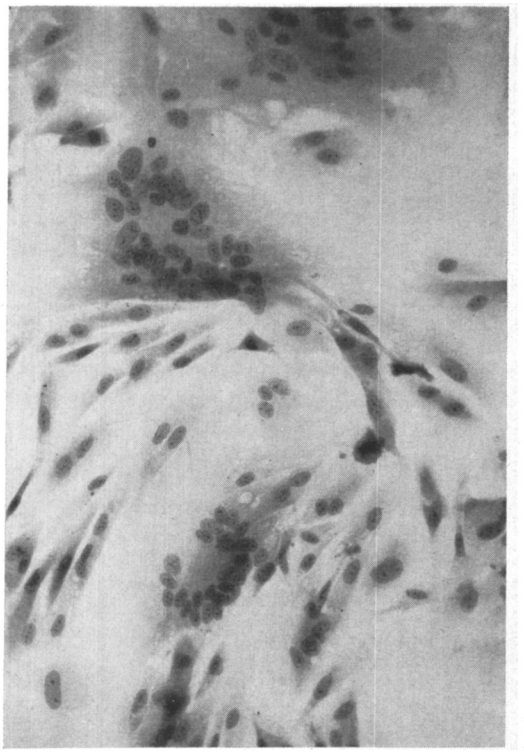

FIG. 3. Cytopathic effect observed 5 days after inoculating normal sheep lung cells with progressive pneumonia virus. Monolayers were inoculated at a multiplicity of 2 $TCID_{50}$ virus/cell. Hematoxylin-eosin stain. (×160).

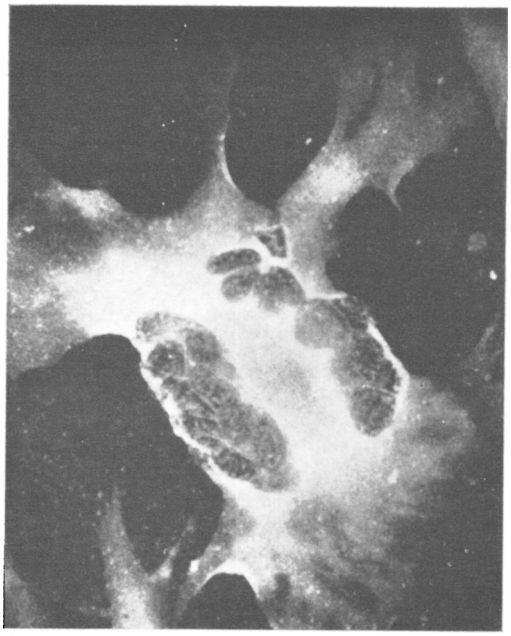

FIG. 4. Immunofluorescent staining of normal sheep lung cells infected with progressive pneumonia virus. Monolayers were inoculated at a multiplicity of 2 $TCID_{50}$ virus/cell and were harvested at 5 days postinoculation. Indirect staining procedure. (×400).

stained by the indirect immunofluorescent technique 3 hr PI.

Specific fluorescence was never observed in uninfected NSL cells tested with sheep antiserum or in infected cells stained with normal sheep serum.

Acridine orange stains double-stranded RNA or DNA green, and single-stranded RNA or DNA red under the staining conditions employed (19,20,21). The red cytoplasmic and green nuclear staining of inoculated and control cultures was similar until 3 days PI. At this time, the cytoplasmic fluorescence in PKC was brighter than in uninoculated cells. This difference increased until 5 days PI, when the cytoplasm of PKC was bright red, whereas the red fluorescence of control cultures was barely visible.

The bright red fluorescence of the cytoplasm was completely abolished by prior digestion with ribonuclease. Incubation with deoxyribonuclease eliminated the green fluorescence of the nuclear chromatin but did not affect the cytoplasmic fluorescence.

Plaque assay. Monolayers were inoculated with serial 10-fold dilutions of tissue-culture fluid containing $10^{6.9}$ $TCID_{50}$ virus/ml. All cells overlaid with carboxymethylcellulose degenerated nonspecifically. Discrete plaques, 1–2 mm in diameter were seen in monolayers overlaid with Noble agar.

The relative efficiency was determined by comparing plaque-forming units, and an estimate of the number of particles determined from the cytopathic end point titer. Tissue-culture infectious units (TCIU) were calculated by multiplying $TCID_{50}$ by 0.69. There were 4.7×10^6 TCIU/ml, and, since 5×10^5 plaque-forming units/ml were observed with the same preparation, the estimate of infectious particles by the end point titration was 10 times higher than that by the plaque assay.

Hemagglutination test. None of the wells showed an agglutination pattern with chick red blood cells.

Hemadsorption test. Hemadsorption pat-

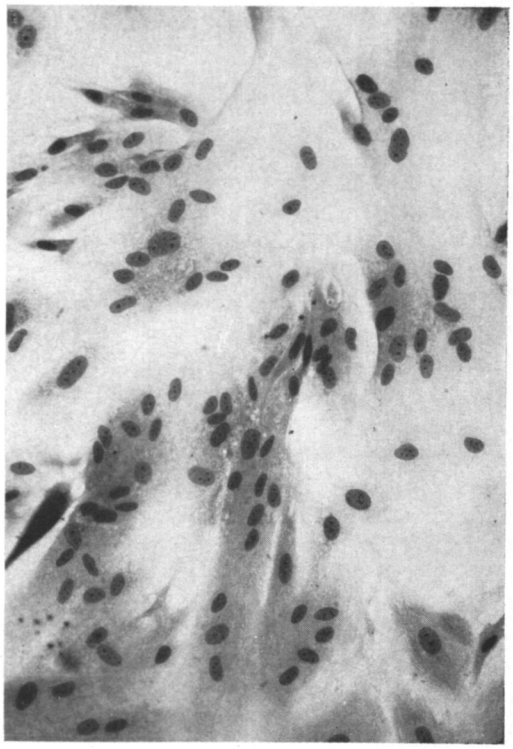

FIG. 5. Cell fusion detected in normal sheep lung cells at 3 hr postinoculation. Monolayers inoculated at a multiplicity of 4 $TCID_{50}$ virus/cell. Hematoxylin-eosin stain. (×160).

terns were not seen with any monolayers infected with PPV when guinea pig red blood cells were used.

Discussion. The clinical signs and pathologic changes of progressive pneumonia are much like those of *maedi* and *zwoegerziekte* (2). Properties of the virus isolated from sheep affected with progressive pneumonia further support the view that these diseases are similar. Thus, growth curves of PPV in tissue culture were similar to those reported for the viruses of *maedi* and *zwoegerziekte* (9, 23). The cytopathic effect, PKC formation, was seen shortly after inoculating monolayers with high concentrations of virus. Bright red cytoplasmic fluorescence of infected cells stained with acridine orange and its abolition by prior digestion with ribonuclease indicate that single-stranded RNA is the nucleic acid of PPV. In addition, this virus did not hemagglutinate chick red blood cells or hemadsorb guinea pig red blood cells. These properties are held in common by the 3 above-mentioned viruses. Recently, Takemoto *et al.* (22) confirmed and extended these results by showing a close serologic relationship between *visna* virus and PPV.

Only slight differences between these viruses have been observed. Harter *et al.* (17) demonstrated brightly fluorescing cytoplasmic inclusions and homogeneous cytoplasmic fluorescence in *visna* virus-infected sheep choroid plexus cells stained by the direct technique. *Visna* virus is closely related to *maedi* virus (23). By the same technique, Thormar (24) observed brightly fluorescing floccules at the cell surface and homogeneous cytoplasmic fluorescence in sheep choroid plexus cells infected with either *visna* or *maedi* virus. In our study, indirect immunofluorescent staining of NSL cells infected with PPV revealed homogeneous cytoplasmic fluorescence similar to that observed with *zwoegerziekte* infected sheep choroid plexus cells (9). These differences might be due to a selection process during tissue-culture passage of the viruses or, as is more likely, to slight differences in host cells used in the 4 studies.

Because only 10% of the estimated number of PPV particles formed plaques, a sensitive plaque assay is not yet available. Nevertheless, some plaques were formed and a plaque assay can probably be developed after the virus has undergone several more passages in tissue culture. A more cytopathic virus population, which will probably develop as the process of selection continues, will enable a more efficient plaque assay to be developed.

Summary. Properties of progressive pneumonia virus (PPV) were studied in sheep lung cell monolayers. Growth studies revealed that, with a multiplicity of infection of 2 $TCID_{50}$ virus/cell, virus replicated to a titer of $10^{6.6}$ $TCID_{50}$ virus/ml in 4 days. Typical polykaryocytes were first seen at 1 day post inoculation (PI) and became larger and more numerous as the virus titer increased. Inoculation of high concentrations of virus produced cell fusion within 3 hr PI. Indirect immunofluorescence revealed homogeneous cytoplasmic fluorescence in infected cells as early as 1 day PI. The number of cells showing fluorescence and the intensity of the fluorescence increased steadily with increase of virus titer. The genetic material for PPV is probably single-stranded RNA. The various properties indicate that PPV is closely related to *maedi, zwoegerziekte,* and *visna* viruses.

1. Duran-Reynals, F., Jungherr, E., Cuba-Caparo, A., Rafferty, K. A., and Helmboldt, C., Ann. N. Y. Acad. Sci **70**, 726 (1958).

2. Marsh, H., "Lung Tumours in Animals," Proceedings of the Third Quadrennial International Conference on Cancer, p. 285. Division of Cancer Research, University of Perugia, Italy (1965).

3. Sigurdsson, B., Grimsson, H., and Palsson, P. A., J. Infec. Dis. **90**, 233 (1952).

4. Koens, H., De "zwoegers" op Texel. Thesis, University of Utrecht (1943).

5. Mitchell, D. T., Union of South Africa, Director Vet. Education and Res. 3rd and 4th Rep., p. 585 (1951).

6. Lucam, F., Recl. Med. Vet. **118**, 273 (1942).

7. Seffner, W., and Lippmann, R., Monatsh. Veterinaer Med. **22**, 901 (1967).

8. Sigurdardottir, B., and Thormar, H., J. Infec. Dis. **114**, 55 (1964).

9. DeBoer, G. F., Zowegerziekte, A Persistent Virus Infection of Sheep. Thesis, University of Utrecht (1969).

10. Kennedy, R. C., Eklund, C. M., Lopez, C., and Hadlow, W. J., Virology **35**, 483 (1968).

11. Merchant, D. J., Kahn, R. H., and Murphy,

W. H., "Handbook of Cell and Organ Cultures," 263 pp. Burgess Publishing Co., Minneapolis (1964).

12. Perry, V. P., Kerby, C. C., Kowalski, F. J., and Malinen, T. I., Cryobiology **1**, 1274 (1965).

13. Dougherty, R. M., *in* "Techniques in Experimental Virology" (R. J. C. Harris, ed.). Academic Press, New York (1965).

14. Liu, C., *in* "Diagnostic Procedures for Viral and Rickettsial Diseases" (E. H. Lennette and N. J. Schmidt, eds.). American Public Health Association, New York (1964).

15. Thormar, H., Acta Pathol. Microbiol. Scand. **68**, 54 (1966).

16. Harter, D. H., Hsu, K. C., and Rose, H. M., J. Virol. **1**, 1265 (1967).

17. Harter, D. H., J. Gen. Virol. **5**, 157 (1969).

18. Hammon, W. McD., and Sather, G. E., *in* "Diagnostic Procedures for Viral and Rickettsial Diseases" (E. H. Lennette and N. J. Schmidt, eds.). American Public Health Association, New York (1964).

19. Gomatos, P. J., and Tamm, I., Proc. Nat. Acad. Sci. U.S.A. **49**, 707 (1963).

20. Gomatos, P. J., Tamm, I., Dales, S., and Franklin, R. M., Virology **17**, 441 (1962).

21. Mayor, H. D., and Hill, N. O., Virology **14**, 264 (1961).

22. Takemoto, K. K., Mattern, C. F. T., Stone, L. B., Coe, J. E., and Lavelle, G., J. Virol. **7**, 301 (1971).

23. Thormar, H., Res. Vet. Sci. **6**, 117 (1965).

24. Thormar, H., Acta Pathol. Microbiol. Scand. **75**, 296 (1969).

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