Onderstepoort J. Vet. Res. (1966), 33 (1), 3–8
Printed in the Republic of S. Afr. by the Government Printer, Pretoria

ELECTRON MICROSCOPIC STUDIES ON LUMPY SKIN DISEASE VIRUS TYPE "NEETHLING"

E. K. MUNZ[1] and N. C. OWEN[2], Veterinary Research Institute, Onderstepoort

The introduction of the negative staining technique has facilitated and considerably improved the electron microscopic demonstration of many viruses. Previously unknown structural details have been revealed and the information on the composition of virions has led to an arbitrary classification of viruses on a morphological basis (Lwoff, Horne & Tournier, 1962).

This technique has also been used extensively in studies on the structure of pox viruses. Horne & Wildy (1963) have reviewed the results obtained by various investigators working with this group of viruses. The more recently published findings of Büttner, Giese, Müller & Peters (1964) and Peters, Müller & Büttner (1964) suggest that the viruses of the pox group can be divided into the following two distinct morphological groups.

(i) Vaccinia-variola virus group;

(ii) *Ecthyma contagiosum* virus (Orf, contagious pustular dermatitis) of sheep; *stomatitis papulosa* virus of cattle and paravaccinia (milkers nodule) virus, for which the name paravaccinia group has been proposed.

Compared with members of the vaccinia-variola group of which the virus particles are brick-shaped with an axis ratio of 1·3, the virus particles of members of the paravaccinia group are smaller and more ovoid, have more pronounced surface filaments, a fainter double element and an axis ratio of 1·7.

Thus far publications on the morphology of the lumpy skin disease virus, type "Neethling", have not been encountered. This virus, shown to be the causative agent of the generally more severe form of lumpy skin disease, is regarded as a member of the pox group. This supposition is based on the occurrence of eosinophilic intracytoplasmic inclusion bodies in infected cells as well as its apparent immunological relationship to sheep pox (Capstick & Coackley, 1961; Weiss, 1963).

In order to confirm the above assumption, an electron microscopic study of the virus has been conducted and the results are presented in this preliminary article.

Materials and Methods

Cell cultures

Primary cultures of calf foetus muscle cells were prepared by overnight trypsinization according to the method of Bodian (1956). The cells were grown at 37° C in Roux flasks in a growth medium consisting of Hanks' salt solution with 0·5 per cent lactalbumin hydrolysate and 10 per cent bovine serum. The medium contained the following antibiotics in the concentrations indicated: Penicillin 100 i.u. per ml, Streptomycin 100 μg per ml, Neomycin 1 mg per ml.

(1) Guest worker from Bayerische Landesimpfanstalt, München, under grant of the Deutsche Forschungsgemeinschaft

(2) Present address: Faculty of Agriculture, University of Natal, Pietermaritzburg.

Received for publication on 29 March 1965.—Editor

Virus

The Neethling type strain (C4L8) originally isolated from a clinical case of lumpy skin disease, was used in these experiments. The strain had been passaged four times in calf kidney and eight times in lamb kidney cell cultures (Weiss, pers. com.).

Virus cultivation

Large quantities of virus were prepared by seeding primary calf foetus muscle cells with this antigen. The medium consisted of Hanks' balanced salt solution with 2 per cent lactalbumin hydrolysate (Weiss & Geyer, 1959). The virus was invariably harvested on the 3rd day, when the cytopathic effect was almost complete. The calf foetus muscle cells were found to yield high virus titers and were therefore suitable for this purpose.

Purification

The purification procedure consisted essentially of a combination of the methods used by Zwartouw, Westwood & Appleyard (1962) and Marquardt, Holm & Lycke (1964) in studies with vaccinia virus. The crude virus bulk (cells and medium) was first homogenized by an " Ultra-Turrax " homogenizer, then treated for 10 minutes in a " Sonblaster " ultra-sonic vibrator operating at 40 KCS. After spinning down the larger particles of debris at 800 g the virus material was pelleted at 35,000 g and resuspended in 0·004 M McIlvaine buffer pH 7·6. The material was then washed twice in this buffer by differential centrifugation at 35,000 g and 800 g respectively and the final deposit resuspended and thoroughly dispersed in 1 : 200 of the original volume.

A sucrose density gradient in McIlvaine buffer was prepared by layering 60, 50, 40 and 30 per cent (w/v) sucrose successively in SW39 Spinco tubes and the concentrated virus carefully floated on top. The tubes were transferred to a SW39L Rotor and spun at 39,000 g for 20 minutes. A small deposit, one clearly visible band (B1) located 6 mm from the bottom and a very faint and narrow band (B2) 21 mm from the bottom of each of the tubes were obtained. The fluid between the bands was clear. Five fractions were aspirated by means of a specially mounted syringe. The first fraction consisted of the fluid above band B2, the second corresponded to band B2, the third was the fluid between the bands, the fourth was band B1, and the fifth consisted of the deposit, resuspended in the fluid above it. In order to remove the sucrose each of the fractions 2 to 5 was diluted 1 : 5 with buffer and pelleted at 35,000 g for 30 minutes. The sediments were resuspended in buffer, again pelleted and each deposit was finally dispersed in 1 ml buffer. In each operation the reagents were kept at $+4^{\circ}$ C as far as possible.

Electron microscopy

Electron micrographs were made on formvar-filmed copper grids or on grids coated with carbon. The preparations were examined in a Philips–EM200 electron microscope. Size calculations were made by photographing diffraction grating replicas for comparative purposes.

Negative staining

Five per cent phosphotungstic acid (PTA) in double distilled water at pH 6·5 to 8·5 was used as recommended by Müller & Peters (1963). The virus was mixed with the stain in equal volumes and then placed on the grids by means of a capillary pipette. In another method, which was also used, the virus was first applied to the grids without any stain. After 2 minutes the grids were washed once with distilled water and then stained *in situ* for 20 seconds with a mixture of 0·5 per cent bovine albumin (1 part) and PTA (2 parts).

All the grids were examined immediately after staining.

Results

The specimens prepared from each of the fractions were examined separately. As in the case of vaccinia (Zwartouw *et al.*, 1962; Marquardt *et al.*, 1964) the majority of the virus particles occurred in the B1 fraction and in the deposit. The B2 fraction consisted mainly of cellular debris as it contained only a few intact particles. The remaining fractions were essentially free of complete virus particles but contained a small amount of debris.

In agreement with the observations made on vaccinia (Müller & Peters, 1963) and on viruses of the paravaccinia group (Peters *et al.*, 1964; Büttner *et al.*, 1964; Nagington, Newton & Horne, 1964) variation in the pH employed with negative staining produced different images. Fig. 1 demonstrates particles found in fraction B1 after staining at pH 6·5. The surface beading is completely irregular. The threads have a width of approximately 70 to 90 Å and form a complex felt work. A regular arrangement of teeth around the margins of some particles was observed. The surface structure therefore appeared to be not unlike those of vaccinia virus photographed under similar conditions and described as type 1 (Nagington & Horne, 1962; Müller & Peters, 1963) or M-forms (Westwood, Harris, Zwartouw, Titmuss & Appleyard, 1964).

Capsular particles, with an outer multilayered membrane or capsule, which has been penetrated by PTA, are seen in Fig. 2 (*a*) and (*b*). These particles appeared to predominate on grids stained at pH 8·5 and corresponded to the C-forms of vaccinia virus mentioned by Westwood *et al.*, 1964 or type 2 of Nagington & Horne (1962) and Müller & Peters (1963). The capsular particles do not show the thread-like structure of the M-forms, but consist of a capsule approximately 280Å thick, surrounding an inner granular homogeneous structure. The capsule itself consists of three definite components, an outer ragged or wavy membrane, a thicker central part and a distinct inner membrane. The arrangement of a triplet-like inner body as well as a double element could not be discovered at this pH. An intermediate form exhibiting a definite capsular membrane with a centrally thickened body was occasionally observed.

Measurements of several particles revealed an average length of 3500 Å and an average width of 3000 Å, giving an axis ratio of approximately 1·2.

At neutral pH conditions both C and M forms were encountered as was found to be the case with other members of the pox group of viruses. Fig. 3 demonstrates both forms of lumpy skin disease virus after staining with PTA at pH 7·0.

Discussion

The classification of the " Neethling " type of lumpy skin disease virus as a member of the pox group is mainly based on the fact that it causes eosinophilic intracytoplasmic inclusion bodies in infected cells and on its apparent relationship to the virus of sheep pox. On the other hand, the biological behaviour of this virus differs in some respects from that of many of the better known pox viruses. For instance, the animal host spectrum of lumpy skin disease virus is very limited, and often difficulty is experienced in the regular artificial production of clinical disease in cattle, the natural host of this virus. Although neutralizing antibodies have been demonstrated in the serum of recovered and vaccinated animals, it has not yet been possible to demonstrate agglutination or agar gel precipitation with the " Neethling " type virus. The virus multiplies in the chorio-allantoic membrane of embryonated eggs but does not produce lesions similar to those caused by other pox viruses.

The results of the electron microscopic investigation reported in this paper, have provided additional evidence in support of the classification of the Neethling type lumpy skin disease virus as a member of the pox group. Depending on the prevailing pH during the negative staining process, two distinct forms of virus particles were revealed. The one is distinguished by irregular threads on its surface, the other by a capsule consisting of three distinct components surrounding an inner body. These features, according to Müller & Peters (1963), are characteristic of pox viruses. The structural appearances of both these forms indicate that the Neethling type lumpy skin disease virus is more closely related to the vaccinia than to the paravaccinia group of viruses. This is further substantiated by the size (approximately 3000 by 3500 Å) and the axis ratio (approximately 1·2) of the particles.

It is therefore suggested that lumpy skin disease virus, type " Neethling ", be considered a member of the pox group, probably belonging to the subgroup vaccinia-variola.

Summary

Electron microscopic studies on lumpy skin disease virus, type "Neethling", have been conducted. Virions which are remarkably similar in size and structural appearance to those of vaccinia virus are described. This is presented as further evidence for classifying lumpy skin disease virus as a member of the pox group.

Acknowledgements

Dr. K. E. Weiss, Head of the Virus Section, is cordially thanked for his invaluable advice and Mr. J. Murphy of the Council for Scientific and Industrial Research for his assistance.

References

BODIAN, D., 1956. Simplified method of dispersion of monkey kidney cells with trypsin. *Virology*, Vol. 2, p. 575.

BÜTTNER, D., GIESE, H., MÜLLER, G. & PETERS, D., 1964. Die Feinstruktur reifer Elementärkörper des Ecthyma contagiosum und der Stomatitis papulosa. *Arch. Virusforsch.*, Vol. 14, p. 657.

CAPSTICK, P. B. & COACKLEY, W., 1961. Protection of cattle against lumpy skin disease. I. Trials with a vaccine against Neethling type infection. *Res. Vet. Sci.*, Vol. 2, p. 362.

HORNE, R. W. & WILDY, P., 1963. Virus structure revealed by negative staining. *Advances in Virus Res.*, Vol. 10, p. 101.

LWOFF, A., HORNE, R. & TOURNIER, P., 1962. A system of viruses. *Cold Spring Harbor Symp. Quant. Biol.*, Vol. 27, p. 51.

MARQUARDT, J., HOLM, S. E. & LYCKE, E., 1964. Preparation of a purified vaccinia virus freed of soluble antigens. *Proc. Soc. Exp. Biol.*, Vol. 116, p. 112.

MÜLLER, G. & PETERS, D., 1963. Substrukturen des Vaccinevirus, dargestellt durch Negativkontrastierung. *Arch. Virusforsch.*, Vol. 13, p. 435.

NAGINGTON, J., NEWTON, A. A. & HORNE, R. W., 1964. The structure of Orf virus. *Virology*, Vol. 23, p. 461.

PETERS, D., MÜLLER, G. & BÜTTNER, D., 1964. The fine structure of paravaccinia viruses. *Virology*, Vol. 23, p. 609.

WESTWOOD, J. C. N., HARRIS, W. J., ZWARTOUW, H. T., TITMUSS, D. H. J. & APPLEYARD, G., 1964. Studies on the structure of vaccinia virus. *J. Gen. Microbiol.*, Vol. 34, p. 67.

WEISS, K. E., 1963. Lumpy skin disease. *FAO Agricultural Studies*, No. 61, p. 177.

WEISS, K. E. & GEYER, S. M., 1959. The effect of lactalbumin hydrolysate on the cytopathogenesis of lumpy skin disease virus in tissue culture. *Bull. Epiz. Dis. Afr.*, Vol. 7, p. 243.

ZWARTOUW, H. T., WESTWOOD, J. C. N. & APPLEYARD, G., 1962. Purification of pox viruses by density gradient centrifugation. *J. Gen. Microbiol.*, Vol. 29, p. 523.

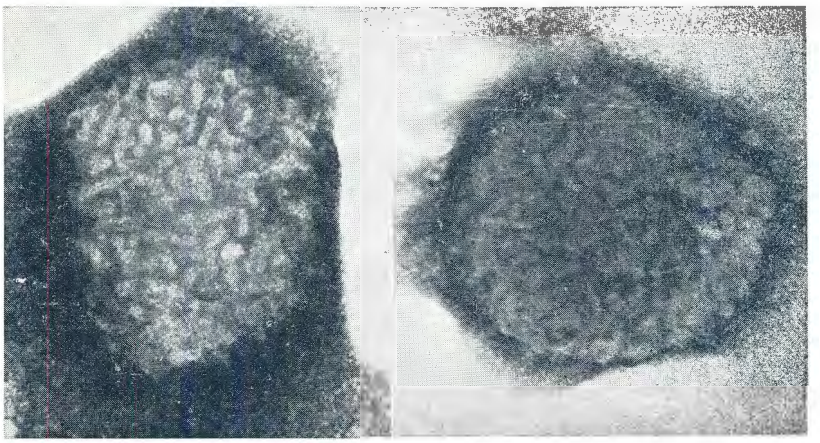

FIG. 1.—Lumpy skin disease virus (" Neethling " type) after staining with PTA at pH 6·5; type 1 or M-forms.
Magnification, 200,000×.

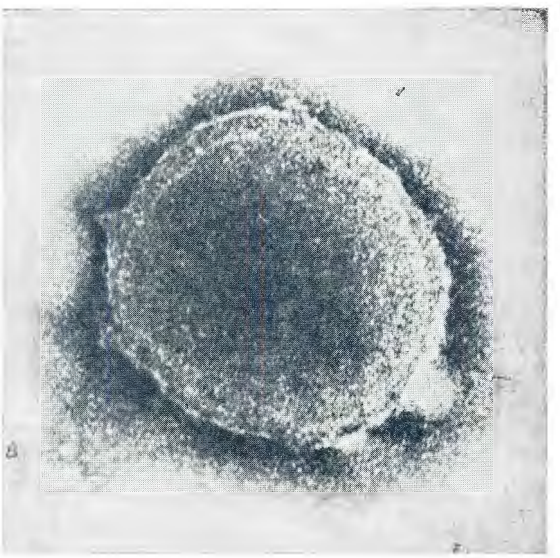

FIG. 2 (*a*).—" Neethling " type of lumpy skin disease virus showing type 2 or C-forms after staining with PTA at pH 8·6.
Magnification 200,000×.

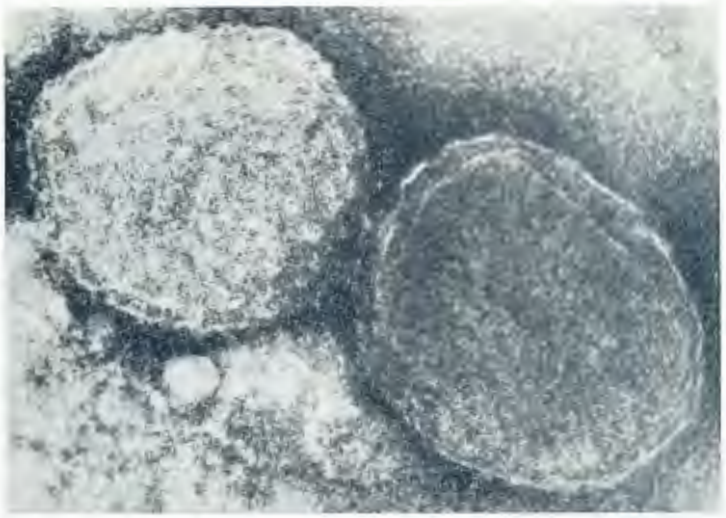

FIG. 2 (*b*).—" Neethling " type of lumpy skin disease virus showing type 2 or C-forms after staining with PTA at pH 8·6.
Magnification, 200,000×.

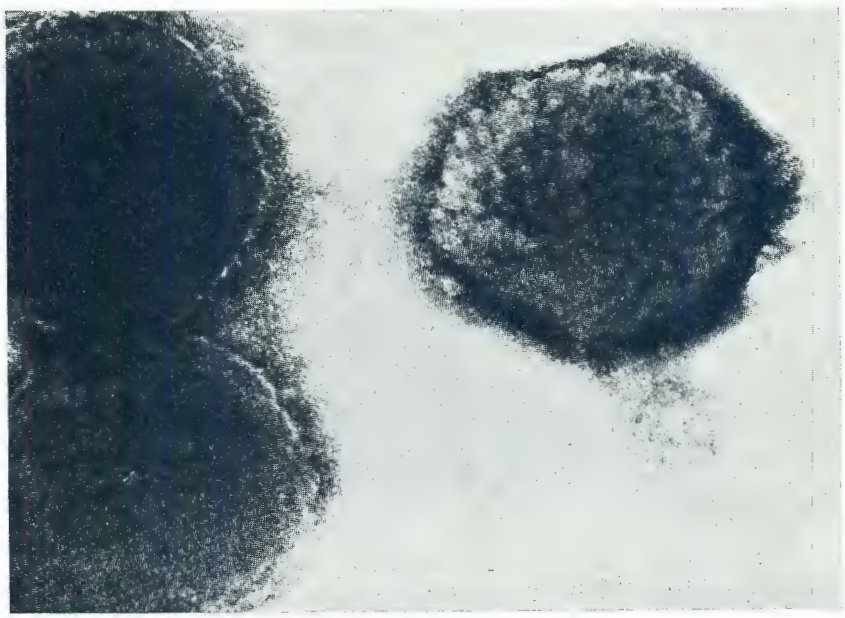

FIG. 3.—Lumpy skin disease virus, " Neethling " type. One particle shows threads (M-forms), two particles with capsules (C-forms). Negative staining with PTA at pH 7·0.
Magnification, 200,000×.