Onderstepoort Journal of Veterinary Research,
Volume 28, Number 2, December, 1959.

The Government Printer, Pretoria.

THE HISTOLOGY OF THE CYTOPATHOGENIC CHANGES PRODUCED IN MONOLAYER EPITHELIAL CULTURES BY VIRUSES ASSOCIATED WITH LUMPY SKIN DISEASE

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The histology of the lesions encountered in Lumpy Skin Disease in cattle was described by Thomas & Maré (1945). They observed intra-cytoplasmic inclusion bodies in histiocytes and epithelial cells in the skin lumps and other affected tissues.

Later Alexander & Haig (1956) succeeded in propagating viruses associated with Lumpy Skin Disease in monolayer cultures of calf kidney cells. They observed cytopathogenesis of the mono- and syncytial type in these cultures. They found inclusion bodies in the nuclei of the syncytia, when stained with haemalum-eosin (Haig, 1957).

Alexander, Plowright & Haig (1957) studied a large number of viruses isolated from skin nodules excised from naturally contracted cases of Lumpy Skin Disease in the Union of South Africa and Southern Rhodesia. They studied the cytopathogenic effects of these viruses on tube cultures and stained coverslip preparations. In this way they were able to divide these viruses into three groups, the cytopathogenesis of which they described.

The method of preparing monolayer cultures on coverslips in Petri dishes for morphological studies was found to be cumbersome and unsuited to the requirements of this exacting study. It was therefore abandoned in favour of a method of preparing specimens from monolayer cell cultures direct from tubes by embedding in collodion, and transfer to a slide for fixation and staining. With minor modifications, a combination of the methods described by Enders & Peebles (1954) and by Reissig, Howes & Melnick (1956) was used with satisfactory results. The advantages of this method are discussed later in this report.

It is the purpose of this paper to describe the sequence of intra-cellular changes occurring in monolayer tube cultures of calf and lamb kidney cells, as well as lamb testis cells, after infection with viruses associated with Lumpy Skin Disease, using the technique to be described.

Material and Methods

Monolayer cultures of calf and lamb kidney, and lamb testis were prepared in tubes according to the technique of Youngner (1954), with minor modifications to meet the requirements of local conditions.

Received for publication on 16 February, 1959.—Editor.

When the cultures were well established a series of tubes was infected with virus isolates representing the three groups described by Alexander, *et. al.* (1957). In order to induce cytopathogenesis to proceed as rapidly as possible high titre virus inocula were used in each case. The tubes were placed in roller drums and incubated at 37° C. They were examined at intervals under low magnification and suitable tubes selected for removal of the monolayer after embedding in collodion. The first tubes were generally removed for examination before cytopathogenic changes were detectable under low magnification and in this way the earliest changes in stained preparations were studied. Depending on the rapidity with which cytopathogenesis progressed, tubes were selected at intervals of from one to several hours and in this way there was accumulated a series of preparations for a detailed study of the cytopathogenesis and its progress.

Various fixation and staining techniques were investigated. Fixation with Carnoy or Zenker fluid, apart from being more laborious, gave no better results than Bouin and, for routine observation, fixation in the latter for one hour was adopted.

A description of the removal of the monolayers from the tubes for fixation and staining on slides now follows.

The culture fluid is withdrawn from the roller tube and the tube rinsed with phosphate buffered saline (PBS) to remove all traces of culture medium. Sufficient Bouin's fluid is poured into the tube to cover the area of outgrowth and left for one hour fixation. After removal of the Bouin, the fixed culture is rinsed with several changes of 70 per cent ethyl alcohol, and may be stored in the latter for an indefinite period. Cultures kept for periods up to one month in 70 per cent alcohol, after fixation, have shown no signs of deterioration. The culture is then dehydrated through 96 per cent alcohol and absolute alcohol to alcohol-ether (50 per cent of each). The latter is then replaced by 4 per cent collodion flexile and this is left in the tube for at least one hour to allow for thorough penetration into the tissue. At this stage the tube may be left overnight, if necessary.

The collodion is poured from the tube, rotating the latter in such a way that the entire inner surface is coated with a thin film. The collodion is then allowed to dry partially, while the tube is rotated in a horizontal position. This requires several minutes until the collodion is set and no longer runs down the side of the tube. The tube is then immersed in cold water, allowing the water to run gently into the tube to harden the membrane. After cutting the collodion membrane at the rim of the tube it is left in water for several more minutes, during which time slight shrinkage of the membrane takes place, whereby it becomes partially detached from the inner surface of the tube. The cut edge of the membrane is then grasped with a pair of slide forceps and the membrane is finally separated from the glass by sliding a smooth glass rod between the two surfaces. The membranous cast is now withdrawn from the tube while the latter is kept under water. The embedded tissue cells are now on the outer surface of the cast. The latter is cut under water into a flat sheet and the portion containing the cell outgrowth is floated onto a glass slide with the surface containing the cells in direct contact with the glass. The film is firmly pressed onto the glass betweeen two layers of filter paper.

The glass slides are then immersed in oil of cloves to clear the collodion membrane and may be left overnight, if necessary. After a contact period of one to several hours, depending on the thickness of the film, the slides are washed in several changes of absolute alcohol and transferred to alcohol-ether for several hours to remove the collodion. The preparations are then stored in 70 per cent alcohol until stained.

Cultures were stained with Haematoxylin (Ehrlich's or Lillie-Meyer) and Eosin (1 per cent aqueous) or Phloxin (1 per cent aqueous). With Haematoxylin-Phloxin staining the changes showed up somewhat better than with Eosin, the inclusion bodies being more intensely stained and giving better contrast for photographic purposes.

In some cases cultures were stained with May-Grunwald and Giemsa, according to the technique of Jacobson & Webb (1952), to show up structures in the inclusion bodies to be described later.

Cultures were also grown in a chamber for continuous observation of living cells by phase microscopy. Initially a chamber was used consisting of a glass slide and glass ring sealed together with a beeswax and vaseline mixture. The cell was filled with fluid medium and covered with a coverslip on which kidney cells previously had been established in a Petri dish. The results were reasonably good but difficulty was experienced in changing the culture medium when it was necessary to carry out observations for a period of several days.

A culture chamber similar to that described by Rose (1954) therefore was made and has been used with excellent results. The chamber consists of two stainless steel plates, each with a circular hole, $\frac{3}{4}$ inch in diameter, through the centre. The two plates, separated by a gum latex rubber gasket, $\frac{1}{8}$ inch in thickness, are held together by four screws, the heads of which are countersunk into one plate. The chamber is completed by coverslips placed on either side of the rubber gasket, sealing off the hole in the latter before the plates are assembled. Tissue cells are previously established on a coverslip in a Petri dish in the usual way and this coverslip is then transferred to the chamber. Culture fluid and virus inoculum are injected into the chamber through a 21-gauge needle piercing the rubber gasket. A second needle, which is left in place, serves as a breather for the free play of air in the sealed chamber when the culture fluid is renewed. The head of this needle is wrapped in sterile aluminium foil to prevent contamination from outside.

Cultures were maintained in this chamber for periods exceeding 14 days, by changing the culture fluid daily, a relatively simple procedure. During the daytime the chamber was mounted under the phase microscope on a heated stage at 37° C for periodic observation and at night it was stored in the incubator room.

Results

A detailed description will now be given of the morphological changes induced by viruses associated with Lumpy Skin Disease, in the same sequence as they were listed by Alexander *et al.* (1957), with the addition of a virus which was isolated subsequent to the above work.

Group 1. Type B.Z.D.

A series of calf kidney cell tube cultures was infected with B.Z.D. virus, generation 8, and slide preparations made of these at suitable intervals, as described above.

1–4.—Cells fixed in Bouins solution, stained with haematoxylin and phloxin, magnification: X750.

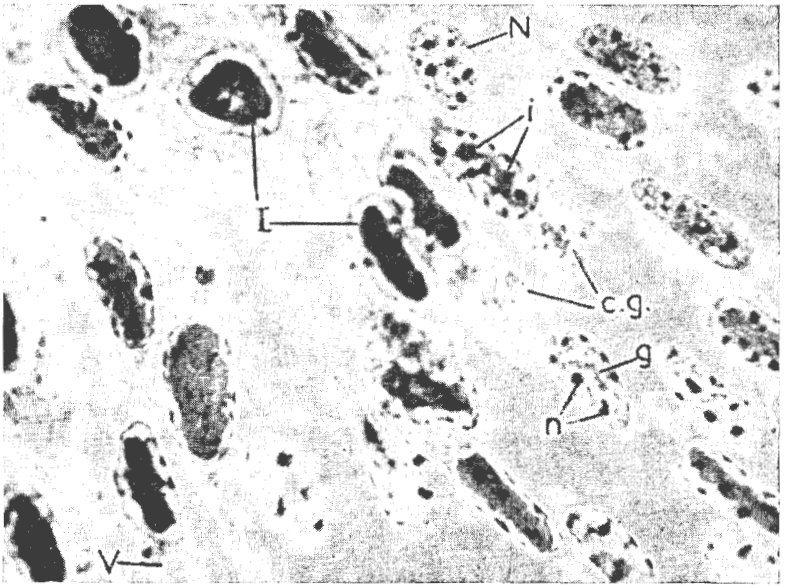

FIG. 1.—Calf kidney cells inoculated with B.Z.D. virus.

c.g.—cytoplasmic granulation
g—acidophilic granules in central area of nucleus—precursors of inclusions
i—condensation of granules forming early inclusions
I—mature inclusions
N—nucleus of normal cell
n—nucleoli
V—cytoplasmic vacuole

A number of cells show Type A inclusions in various stages—note variations in density, width of halos, and margination of chromatin.

At 30 hours after virus inoculation the first changes could be detected in the cells, in the form of fine eosinophilic granules scattered haphazardly in the chromatin network of the nuclei. (Fig. 1.) As the granules grew in size and number they became consolidated into larger masses, from which the chromatin material receded, with the result that the masses were surrounded by a clear zone in the form of a halo which did not stain with Haematoxylin and Phloxin. The eosinophilic masses eventually coalesced to form one solid body occupying the entire central portion of the nucleus. As this body grew in size the chromatin receded towards the nuclear membrane, eventually becoming completely marginated. The body conformed in every way to Cowdry's (1934) description of Type A inclusions. The nuclear membrane, with the marginated chromatin clearly outlined, became wrinkled and constricted round the central inclusion, with the result that the halo became progressively narrower and eventually disappeared. In the earlier stages the inclusions were distinctly acidophilic but later became more dense in structure and assumed a slatey, bluish-grey colour with a tendency to appearing basophilic. Some of the larger inclusions appeared to break up into a number of closely packed refractile bodies, giving the inclusion

the appearance of a honeycomb network. While the above changes were taking place in the nuclei, the cytoplasm became more dense and acidophilic, with the formation of granules and occasional vacuoles. The entire cell became shrunken and rounded or elongated. Irregular holes developed in the monolayer as a result of this shrinkage. The affected cells gradually detached themselves from the glass surface. The nuclei assumed various bizarre shapes, from multilobular to crescent-shaped, staining more deeply as pyknosis progressed. Eventually the chromatin broke up into numerous fragments in a process of karyorrhexis, destruction of the monolayer being complete in about six days.

The morphological changes in lamb kidney cells were essentially the same as in the calf kidney cells. The changes described were typical for all other isolates of this group studied.

Group II. Type Allerton.

As above, a series of tube cultures were inoculated with Allerton virus, generations 9 and 62, respectively. As development in cultures of this group of virus takes place very rapidly, tubes were selected and slide preparations made at two-hourly intervals throughout a period of 24 hours.

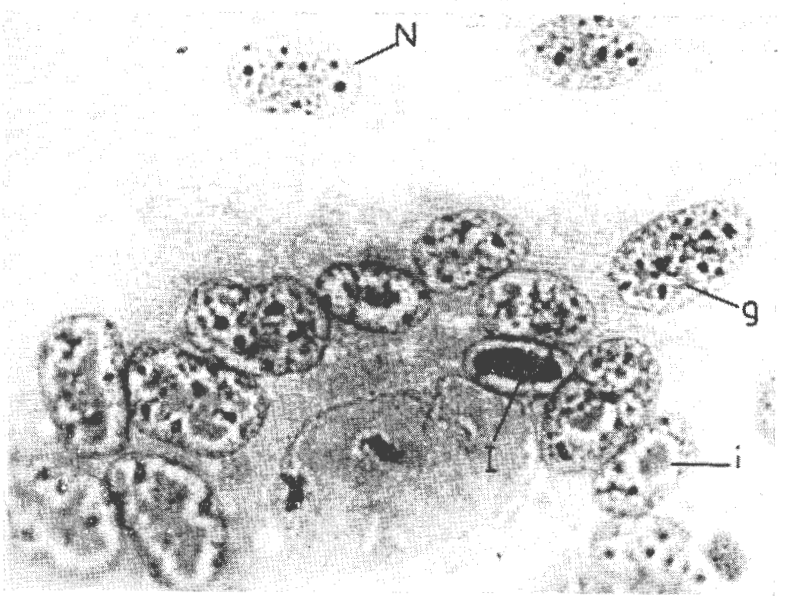

FIG. 2.—Lamb kidney cells, inoculated with Allerton virus.
g—acidophilic granules—precursors of inclusions
i—early inclusion body
I—mature inclusion body
N—nucleus of normal cell

All the cells in the lower two-thirds of the figure are part of a multi-nuclear syncytium and show various stages in the formation of Type A inclusions. Note variations in density, width of halos, margination of chromatin and cytoplasmic granulation.

At four hours early indications of the formation of intranuclear inclusions were detectable. Acidophilic granules were forming, increasing in size and numbers (Fig. 2), in a manner very similar to those seen in Group I above, eventually becoming coalescent and forming large type A inclusions. Generally, they appeared more dense and massive than the Group I inclusions and development proceeded much more rapidly. The staining reaction of the inclusions also tended to become basophilic with progressive development as in the case of the Group I inclusions. Margination of the nuclear chromatin took place as the inclusions increased in size and the latter were surrounded by a halo, which became narrower as the nuclear membrane contracted round the inclusion.

While the above changes occurred in the nuclei, the cytoplasm became progressively more acidophilic and the lines of demarcation between adjoining cells disappeared. The cytoplasm of adjoining cells became confluent and in this way large multinuclear syncytia were formed. Isolated cells, which could not participate in the formation of syncytia, became rounded, shrunken and partially detached from the glass surface.

In the process of shrinkage and syncytia formation some of the cells became drawn out into elongated strands of cytoplasm, with correspondingly elongated nuclei, stretching across the holes formed in the cell sheet.

Eventually the syncytia developed into spherical masses which became detached from the glass surface and floated away in the fluid medium. Destruction of the monolayer was completed in 24 hours.

All the above changes were followed by continuous observation of living cells in the tissue culture chamber by phase microscopy.

It was noted that the changes progressed somewhat more rapidly in the cultures infected with Allerton, Generation 9, than in those infected with Generation 62. Again the changes described were typical for all other isolates of this group.

Group III. Type Neethling.

Separate isolates of viruses of this group were inoculated on lamb kidney cells in culture tubes and slide preparations were made at suitable intervals. The cytopathogenic changes produced by each virus were essentially the same and could be regarded as identical for the purpose of this study.

At 45 hours intracytoplasmic bodies became evident. At first they were small, rounded, somewhat basophilic and surrounded by a clear halo. (Fig. 3.) As they increased in size they became distinctly acidophilic, the outline became irregular and there developed a central, basophilic " inner body ". Stained with Giemsa and examined by phase microscopy, these inner bodies assumed a bright blue coloration believed to be due to ribonucleoprotein. (Jacobson & Webb, 1952.)

In the earlier stages the cytopathogenic changes occurred in distinct " plaques " or clusters of cells, from where they gradually invaded the surrounding monolayer.

All the progressive stages of cytopathogenesis therefore could be detected in one plaque, the more advanced changes occurring in the centre, while the more recent changes were found to take place along the periphery of the plaques.

As the inclusions increased in size the nuclear membrane became wrinkled and an indentation appeared, usually on the side nearest the inclusion body. The degenerating cells contained one or more inclusions.

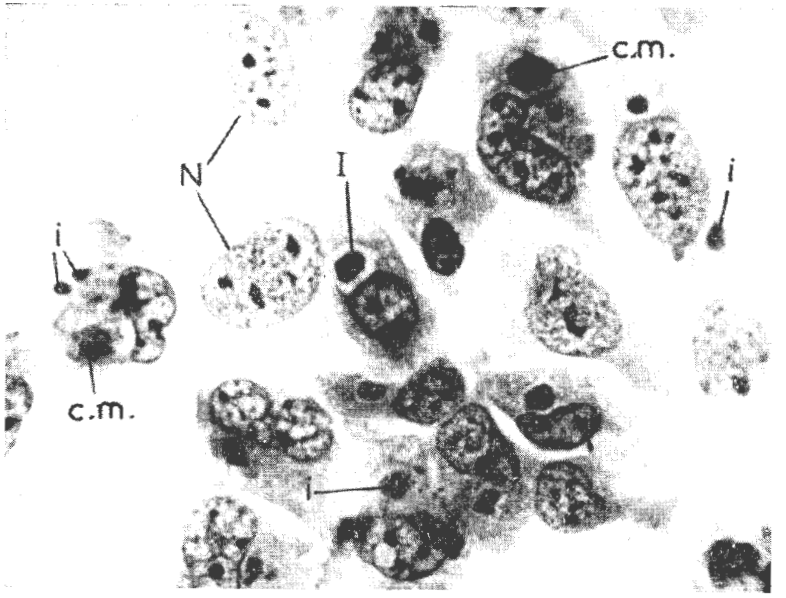

FIG. 3.—Lamb kidney cells, inoculated with Neethling virus.

c.m.—acidophilic cytoplasmic mass
i—early intracytoplasmic inclusions showing basophilic "inner bodies"
I—mature inclusion with halo
N—nuclei of normal cells

Note wrinkling, shrinkage and indentation of nuclei of affected cells, and increase in density of cytoplasm.

At about 60 hours numbers of cells became shrunken and rounded, leaving large, irregular holes in the cell sheet. The cytoplasm became progressively more acidophilic, while in the large inclusions the basophilic inner body broke up, becoming dispersed in the inclusion and assuming a frothy appearance. At the same time, an intensely staining eosinophilic "mass" developed in the cytoplasm near the inclusion, while the nucleus became more and more indented, progressively pyknotic, wrinkled, and distorted into various shapes, and was pushed towards the edge of the cytoplasm. The eccentric nucleus in many cases became crescent-shaped.

Eventually the inclusion body appeared to break up and merge with the eosinophilic mass, leaving only traces of basophilic material to indicate its former existence.

At 114 hours a considerable portion of the monolayer was destroyed, large numbers of cells having become rounded and partially detached, while the nuclei became progressively more pyknotic. The process of karyorrhexis, characteristic of the final stages of cytopathogenesis in Groups I and II, was rarely seen in the case of Group III.

There was no evidence of the formation of syncytia.

Destruction of the monolayer was complete at about 130 hours.

Similar changes occurred in calf kidney and lamb testis cell cultures, but the process appeared to be more rapid in the latter.

The above cytopathogenic effects could be closely followed in living cells under the phase microscope. In this the larger inclusions appeared to consist of numerous closely-packed, semi-refractile bodies.

An Ecbo Virus

During the course of recent experimental observations on the above viruses, a virus was isolated from the faeces of an experimentally infected bovine (Alexander, Weiss & De Lange, 1958).

The original material produced a rapidly developing cytopathogenesis when inoculated on lamb testis cell cultures, and the following morphological changes were noted:

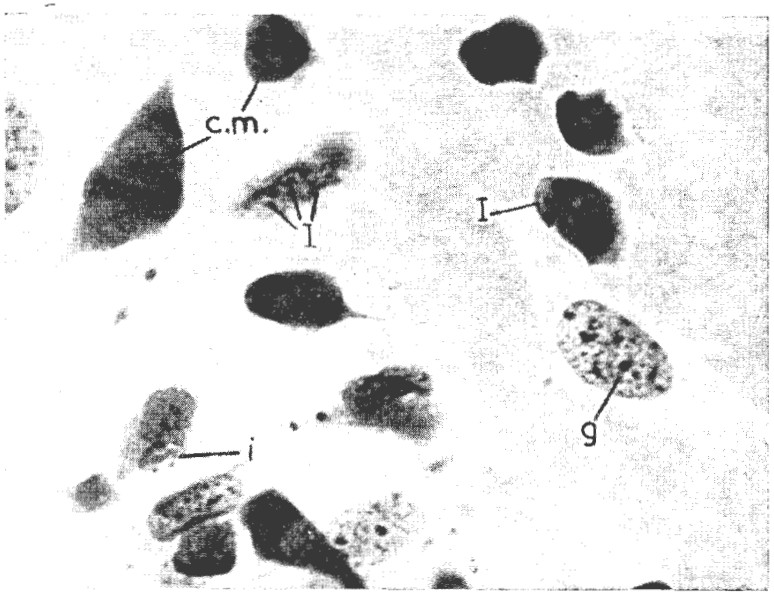

FIG. 4.—Lamb kidney cells inoculated with a virus isolated from bovine faeces.

c.m.—acidophilic cytoplasmic mass
g—acidophilic granules—precursors of inclusions
i—early inclusion with halo
I—mature, contracted Type B inclusions in distorted and shrunken nuclei

Note crescent shape of eccentric nuclei and increased density of cytoplasm in cells showing advanced cytopathogenic changes.

The earliest detectable change took place in the nucleus in which there was loss of chromatin usually in the central area, with the appearance of fine acidophilic granules in the denuded areas. These granules soon condensed into more intensely stained inclusion bodies, one or more per cell, the shape of which followed the contours of the denuded areas and were surrounded by a distinct

halo (Fig. 4). At the same time the nuclear membrane became wrinkled and the nucleus assumed a more elongated shape. A condensation of chromatin took place near the edges of the nuclear membrane, while the nucleoli could be clearly distinguished from the inclusion bodies by their basophilic staining and the absence of a surrounding halo.

As the nucleus became progressively more wrinkled and shrunken, the nucleoli merged with the basophilic chromatin and no longer could be distinguished from the latter. At the same time the inclusions became more dense and contracted, assuming a rounded outline with a clear halo separating them from the surrounding basophilic material. They could be classified as Cowdry Type B inclusions.

While the nucleus contracted the cytoplasm became eosinophilic and the nucleus was pushed aside by the formation of an acidophilic cytoplasmic mass. The cell became rounded and partly detached from the glass surface. The pyknotic nucleus became distorted and assumed various shapes as it was pushed towards the cell margin by the cytoplasmic mass, usually ending up as a deeply basophilic, crescent-shaped body along the periphery of the cell. In many of these crescent-shaped, pyknotic nuclei one or more small, rounded inclusions remained visible, lying in spaces cleared of chromatin and surrounded by a halo.

During the process of contraction of the cells, some showed the presence of cytoplasmic vacuoles of varying size and number, but these were not constantly present in all cells. In the final stages the small, rounded cells became completely detached from the glass and floated away in the fluid medium.

In the process of contraction and detachment of the cells, irregular holes appeared in the cell sheet, similar to those seen in the previous groups. Destruction of the monolayer was complete in some cultures as early as 16 hours after virus inoculation.

The virus appeared to multiply equally well on lamb kidney and testis cells, and on calf kidney cells.

Discussion

When observing the morphological changes induced in tissue cultures by the viruses associated with Lumpy Skin Disease, one is struck by the remarkable similarity in the formation of intranuclear Type A inclusions by the Group I and II viruses. The cytopathogenic changes in these two groups differ only in the formation of syncytia in Group II, which is absent in Group I. In this way the two groups may be differentiated apart from the fact that cytopathogenesis proceeds more rapidly in Group II than in Group I.

The changes induced by the Group II viruses are very similar to those produced by cytopathogenic agents recovered from cases of measles as described by Enders & Peebles (1954) namely the formation of large intranuclear inclusions, margination of chromatin, and formation of syncytia. On the other hand Sherman & Ruckle (1958) described small intranuclear, as well as cytoplasmic, inclusions in the syncytia found in lung cultures from cases of measles.

The intracytoplasmic inclusion bodies found in tissue culture preparations of Group III viruses are very similar to those described by Thomas & Maré (1945) in the epithelial and histiocytic cells of the skin in Lumpy Skin Disease. Alexander *et al.* (1957), mention the resemblance of these bodies to those produced by vaccinia and other members of the pox group of viruses.

Attention is directed to the close similarity of the cytopathogenic changes induced by the virus isolated from bovine faeces, and those described by Reissig, Howes & Melnick (1956) in monolayers of monkey kidney cells infected with polio virus. This suggests that, on morphological grounds at least, these viruses belong to the same group. The significance of this virus in the aetiology of Lumpy Skin Disease is at present obscure. It is possible that it is merely an enteric cytopathogenic bovine orphan (Ecbo) virus similar to those described by Kumin & Minuse (1958).

At this stage it is expedient to discuss the marked advantages of the technique used for the preparation and staining of the material for histological examination in this study. The underlying principle is the removal of the monolayer of cells intact from a test tube after examination under low magnification and its transfer for fixation upon the flat surface of a glass slide for examination under high power magnification after suitable staining.

The simplicity of the technique makes it possible to examine large numbers of preparations and it is remarkable how little distortion of the cells of the cell sheet takes place. It is appreciated that equally beautiful slides may be prepared from coverslip cultures specially prepared and infected for the purpose. However, this procedure necessitates subculture and there is no guarantee that the cytopathogenesis induced in the sub-culture is identical with that observed in the original monolayer tube. This point is strikingly illustrated by a recent occurrence in these laboratories.

A skin nodule from a naturally contracted case of Lumpy Skin Disease was submitted for examination. As a routine procedure six calf kidney epithelium monolayer tubes were seeded with the prepared inoculum, no serum being used in the nutrient medium. In due course cytopathogenesis developed which, on histological examination indicated the presence of Group I and III viruses. This finding was confirmed by the production of antibodies against Group III virus in a susceptible bovine which received an injection of portion of the harvested cultures. On tissue culture sub-culture, the Group I virus outgrew the Group III virus with the result that only Group I cytopathogenesis could be demonstrated histologically. The accuracy of this observation was confirmed by the failure of a second susceptible bovine to produce Group III antibodies after injection of the sub-cultured inoculum.

In addition it is our experience that seldom is it possible to set up parallel tube and coverslip preparations. It has happened that only one of a series of tubes may show a particular change. In that case it has been possible to use the culture fluid and portion of the monolayer for subculture while the balance of the monolayer could be used for detailed histological examination. The value of such a procedure needs no emphasis but it may be mentioned that it has enabled us rapidly to make a tentative diagnosis of Rift Valley Fever virus infection and without delay to set up the appropriate serum virus neutralization tests to confirm that tentative diagnosis.

Finally it should be pointed out that after being afforded an opportunity of working with these viruses in what are believed to be pure culture, it has been possible to refer back, not only to the published descriptions of the pathology of Lumpy Skin Disease in cattle but also, in some instances, to the actual material examined at that time. The result has been that many controversial points have been cleared up, points associated with the specific changes produced by viruses of the different groups *in vivo* such as the formation of syncytia, the identification of intranuclear and intracytoplasmic inclusions, the nature of the inflammatory reactions and the tissues involved. It is evident that the published descriptions of the histology of Lumpy Skin Disease in cattle may require critical review in the light of our present knowledge.

Summary

A study is reported of the cytological changes induced in cultures of calf and lamb kidney cells and lamb testis cells by three distinct groups of viruses associated with Lumpy Skin Disease. On the basis of the morphological changes produced one is justified in concluding that these viruses are entirely unrelated. The cytopathogenesis of a virus isolated from bovine faeces is also described.

Attention is directed to the similarity of cytopathogenic effects of these viruses to those induced by other viruses causing known disease conditions.

The value of the histological study of cytopathogenesis in relation to virus research is discussed.

Acknowledgement

Thanks are due to the Director of Veterinary Services for providing the facilities and material required for this study, and for granting permission to publish this report.

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