The Neural Spread of Pseudorabies Virus in Calves

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SUMMARY

The neural spread of pseudorabies virus in calves is described. Twenty-four calves were infected subcutaneously with the virus and killed from 48 h after inoculation until the terminal stages of the disease at 120 h post-inoculation. Virus tissue isolations and histological, fluorescent and electron microscopic techniques were employed.

Virus multiplied in the fasciae at the inoculation site for 60 h and subsequently appeared almost simultaneously in the entire 75 cm length of peripheral nerve, and related spinal ganglion and spinal cord segment. As the disease progressed virus spread cranially and caudally along the spinal cord so that by death virus was present throughout the central nervous system. The findings were indicative of a centripetal spread of virus along the peripheral nerve.

The studies established that a cellular progression of virus to the central nervous system was not possible. The pathway of the virus must have involved a fluid medium since it travelled over 75 cm in less than 72 h. The media available were the perineurial fluid, endoneurial fluid and axoplasm. No evidence of virus transport in the perineurial fluid was observed, and although some movement probably occurred in the endoneurial fluid, extracellular virus was not seen in either the nerve or ganglion. Both naked and enveloped virus particles were seen in the axoplasm of nerve fibres throughout the peripheral nerve and ganglion following replication in ganglionic neurons but virus particles were not identified during the incubation period. The passage of one or two infecting particles during the latter period, however, would be very difficult to detect by all the techniques employed. Evidence is presented for the axoplasm as the pathway of the virus to the spinal ganglion and central nervous system.

INTRODUCTION

The routes and mechanisms by which viruses gain access to the central nervous system of man and animals have presented many problems to pathologists and virologists for the past century (Wright, 1953; Johnson & Mims, 1968). Although many viruses gain access to the central nervous system via the blood, inoculation of experimental animals with herpes viruses only rarely results in a viraemia (McFerran & Dow, 1964, 1965; Wildy, 1967).

It is now generally accepted that herpes simplex and pseudorabies viruses travel to cranial or spinal ganglia and the central nervous system along peripheral nerves, but controversy exists as to the neural structures involved (Hurst, 1933; Johnson, 1964; Wildy, 1967; Kristensson, Lycke & Sjostrand, 1971).

The present work was undertaken to study the development and pathogenesis of
pseudorabies in calves and to attempt to define the pathway of the virus in the nerve. It was considered that such an attempt should simultaneously utilize virus tissue titrations and histological, fluorescent and electron microscopic techniques at all stages of the disease, so that all possible forms of the virus could be detected and recognized in nervous tissue.

METHODS

Virus. The virus used was a 5th pig kidney tissue culture passage originally isolated from a naturally occurring case of pseudorabies in a pig. This strain, the Northern Ireland A1 (N.I.A.1), is strictly neurotropic in the calf (Dow & McFerran, 1962, 1966; McFerran & Dow, 1964). On experimental inoculation this virus produces a uniformly fatal disease in calves with an incubation period of 72 to 100 h. The onset of clinical signs is characterized by intense local pruritus at the site of inoculation and death occurs 24 to 48 h later. A single pool was grown in pig kidney cell culture to a titre of $10^{7.0}$ tissue culture infective doses 50 (TCID$_{50}$/ml).

Experimental infection. A total of twenty-four 3-month-old Ayrshire male calves was used. Pre-infection sera from the calves were tested for evidence of pseudorabies antibodies using the serum neutralization test and all were consistently negative. Eight calves were used in the combined fluorescent antibody and virus isolation experiments and of these two were killed when moribund at 120 h post-inoculation (p.i.), and the remaining six at 108, 96, 84, 72, 60 and 48 h p.i. Sixteen calves were used in the ultrastructural studies, five being killed at 120 h p.i., four at 108 h p.i., two each at 96 and 84 h p.i., and one each at 72, 60 and 48 h p.i. Calves were infected subcutaneously behind the 10th left costo-chondral junction with 1.0 ml of virus suspension. It was considered essential to locate an area of skin which was innervated by nerve fibres derived from a single nerve trunk and associated spinal ganglion and the innervation of the posterior thoracic wall (Kuz'minov, 1953) was judged to fulfil these criteria most efficiently. Furthermore, the length of nerve from the inoculation site to the spinal ganglion was approximately 75 cm and so adequate nerve tissue was available for the various examinations.

Virus isolation. The calves were killed with intravenous pentobarbitone sodium and at autopsy the following specimens were collected for virus isolation: anterior cerebrum, cerebellum, medulla oblongata, 2nd, 4th and 6th segments of cervical spinal cord, 2nd, 4th, 6th, 8th, 10th and 12th segments of thoracic spinal cord, 2nd, 4th and 6th segments of lumbar spinal cord, 10th left thoracic spinal ganglion, 10th left thoracic spinal nerve roots, five portions of the 10th left intercostal nerve (N1 that adjacent to the ganglion and N5 that at the inoculation site), superior cervical sympathetic ganglia, pooled thoracic sympathetic ganglia, adrenal gland, heart, liver, spleen, lung, kidney, blood collected in acid citrate dextrose solution and portions of skin, underlying fasciae and muscle from the inoculation site. Samples of the organs were prepared and inoculated immediately into primary pig kidney tissue culture. The presence of the virus was confirmed by the neutralization of the isolate by antiserum to pseudorabies virus.

Fluorescence microscopy. Tissues examined by fluorescence microscopy included skin, muscle and fasciae from the inoculation site, five portions of the 10th left intercostal nerve, and the corresponding spinal ganglion, nerve roots and spinal cord segment. Similar control tissues were taken from uninfected calves. Frozen sections were fixed in acetone at $-20^\circ$C for 30 min and subsequently stained overnight at 4 $^\circ$C using the direct immuno-fluorescence technique. Antiserum was prepared in pigs by intranasal inoculation of N.I.A.1 virus. Immune and pre-immune sera were conjugated with fluorescein isothiocyanate by the
Table 1. Distribution of virus following inoculation of calves with the N.I.A. 1 strain of pseudorabies virus

<table>
<thead>
<tr>
<th>Tissue</th>
<th>48</th>
<th>60</th>
<th>72</th>
<th>84</th>
<th>96</th>
<th>108</th>
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<tbody>
<tr>
<td>Anterior cerebrum</td>
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<td>Mesencephalon</td>
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<tr>
<td>Cerebellum</td>
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<td>Medulla oblongata</td>
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<td>2nd cervical</td>
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<td>4th cervical</td>
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<tr>
<td>2nd thoracic</td>
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<tr>
<td>6th thoracic</td>
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<tr>
<td>10th thoracic</td>
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<td>2nd lumbar</td>
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<td>6th lumbar</td>
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<td>10th nerve roots</td>
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<tr>
<td>10th spinal ganglion</td>
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<td>N1</td>
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<td>N2</td>
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<td>N3</td>
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<td>N4</td>
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<td>N5</td>
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<td>+</td>
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<tr>
<td>Skin</td>
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It should be noted that virus was not recovered from visceral organs or blood excepting the adrenal glands, heart and autonomic ganglia.

Method of Todorov, Wilkinson & White (1968). Samples of unlabelled immune and pre-immune sera were also held for use in control tests.

Electron microscopy. The sixteen calves were anaesthetized and perfused with buffered glutaraldehyde as previously described (McCracken, 1972). After perfusion was completed the 10th left thoracic spinal ganglion and intercostal nerve were identified, carefully dissected and processed. Each specimen was cut into blocks approximately 1 mm³, placed in 4% glutaraldehyde at 4 °C for 2 h and postfixed in 1% osmium tetroxide. The blocks were embedded in Araldite and sections stained with uranyl acetate and lead citrate.

Light microscopy. Material from all 24 calves was taken for histological examination. Tissues similar to those for virus isolation were taken, fixed in 4% buffered glutaraldehyde for 48 h at room temperature and processed by standard paraffin techniques.

Results

Virus isolations

In the calf killed at 48 h p.i., virus was isolated from the fasciae beneath the inoculation site only and at 60 h p.i. was also present in two sites in the nerve (Table 1). In the calf killed at 72 h p.i. virus was recovered from the subcutaneous fasciae, the proximal half of the intercostal nerve and the 10th thoracic segment of spinal cord.

The calf killed at 84 h p.i. had been exhibiting pruritus for approximately 5 h prior to killing and virus was present in all regions of the intercostal nerve, in the 10th thoracic spinal ganglion, in the 10th left thoracic nerve roots, and in the spinal cord from the 2nd thoracic segment to the 2nd lumbar segment (Table 1). At all later stages of the disease virus was almost invariably recovered from the intercostal nerve, spinal ganglion, nerve roots and spinal cord. Subsequent to 84 h p.i. virus spread cranially and caudially along the spinal
Table 2. Distribution of virus antigen by fluorescence microscopy following inoculation of calves with the N.I.A. 1 strain of pseudorabies virus

<table>
<thead>
<tr>
<th>Tissue</th>
<th>48</th>
<th>60</th>
<th>72</th>
<th>84</th>
<th>56</th>
<th>108</th>
<th>120</th>
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<tbody>
<tr>
<td>10th thoracic</td>
<td></td>
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<td>+</td>
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<td>++</td>
<td>++</td>
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<td>10th nerve roots</td>
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<td>10th spinal ganglion</td>
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<td>10 N1</td>
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<td>10 N2</td>
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<td>10 N3</td>
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<td>10 N4</td>
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<td>Skin</td>
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</table>

cord so that by the terminal stages of the disease all segments of the cord contained infective virus. Virus was also isolated from the brains of both moribund calves.

In the later stages of the disease virus was also recovered from sympathetic ganglia, adrenal gland and in one of the moribund calves from the heart. No other tissues yielded virus at any stage of the disease.

Fluorescence microscopy

Virus antigen was detected only in fibroblasts and macrophages in the fasciae at the inoculation site of calves killed at 48 and 60 h p.i. (Table 2). In the calf killed at 72 h p.i. specific fluorescence was also demonstrated in the 10th left thoracic spinal ganglion and associated dorsal horn of spinal cord. At all subsequent stages virus antigen was invariably detected in all portions of the nerve and in the associated spinal ganglion, nerve roots and segment of spinal cord. As the disease progressed the intensity of fluorescence in cells of the fasciae at the inoculation site became weaker, so that by 72 h. p.i. only an occasional cell contained virus antigen.

In sections of intercostal nerve specific fluorescence was detected in the endoneurium from 84 h p.i. (Fig. 1), but was confined to a few cells in any one section. The reaction was almost entirely restricted to Schwann cells (Fig. 2) and was observed in both the nucleus and cytoplasm. Virus antigen was not seen in axons. Specific fluorescence was not detected in the perineurium and the only evidence of virus antigen in the epineurium was the presence of fluorescing material in the cytoplasm of an occasional macrophage. The distribution of virus antigen in nerve roots was similar to that in the peripheral nerve.

At 72 h p.i. a few ganglionic neurons displayed nuclear fluorescence, but at subsequent stages intense fluorescence was observed in both the nucleus and cytoplasm of many neurons (Fig. 3). Virus antigen was never detected in the surrounding satellite cells but occurred randomly in ganglionic Schwann cells.

At 72 h p.i. specific fluorescence was detected in the apex of the left dorsal horn of the 10th thoracic spinal cord segment and was limited to the nuclei and cytoplasm of small neurons. At 84 h p.i. antigen was more prominent in the left dorsal horn and was now present in the left ventral horn and right dorsal horn of the spinal cord. Fluorescent material was generally present in both the nucleus and cytoplasm of affected neurons. In the later stages fluorescence was widespread throughout the grey matter but was most intense in the left dorsal horn. Virus antigen was not observed in the vascular endothelium, ependymal cells nor in the meninges.
Fig. 1. Fluorescing material is present in the endoneurium (N) of a nerve bundle. P = epineurium.
Fig. 2. Virus antigen is present in Schwann cells but not in the adjacent axons (A). N = endoneurium, P = epineurium.
Fig. 3. A ganglionic neuron contains virus antigen in the cytoplasm and nucleus.
Fig. 4. Section of ganglion showing two basophilic intranuclear inclusion bodies (arrows). Neuronal degeneration and neuronophagia are seen. Haematoxylin and eosin.

Fig. 5. An unenveloped virus particle (arrow) is present within an unmyelinated axon (A). S = Schwann cell.
Fig. 6. Two virus particles (arrows) budding at smooth-membraned vesicles in the axoplasm. M = myelin sheath.

Fig. 7. Section of myelinated axon. Enveloped virus particles (arrows) are present in vesicles in the axoplasm. M = myelin sheath.
Histological changes were detected in the fasciae underlying the inoculation site at 48 h p.i., but lesions were not observed in any of the neural tissues during the incubation period. In calves killed at 84 h p.i. evidence of virus infection was present in the spinal ganglion and 10th thoracic segment of spinal cord, and consisted of neuronal degeneration and basophilic intranuclear inclusion body formation (Fig. 4). At all subsequent stages histological evidence of virus infection was observed in all the neural tissues associated with the inoculation site.

Lesions in the spinal cord were initially focal and as the disease progressed the foci coalesced to produce a generalized neuronal degeneration. In contrast, lesions in the spinal ganglia remained focal throughout the course of the disease. At an early stage of the disease only a few infected neurons were evident and, as the disease progressed, although more and more neurons became infected they were still generally separated from each other by numerous normal neurons. The satellite cell layer surrounding infected neurons remained intact during the entire process of neuronophagia and there was no evidence of lateral spread of infection. Infected neurons were randomly distributed throughout the ganglion and a concentration of infected cells was not apparent at the hilus.

**Electron microscopy**

Virus particles were first observed 84 h after infection and were seen only in the nucleus and cytoplasm of isolated ganglionic neurons. At all subsequent stages virus was invariably observed in the spinal ganglion and in all regions of the intercostal nerve.
Nerve

From 96 h p.i. virus particles were occasionally seen in the axoplasm of myelinated and unmyelinated nerve fibres at all levels of the nerve but were especially obvious in moribund calves. Naked capsids were seen free in the axoplasm (Fig. 5) or budding into smooth-membraned vesicles (Fig. 6) and enveloped particles were also seen within similar vesicles (Fig. 7). Virus was occasionally observed within isolated Schwann cells which were randomly distributed and were never associated with infected axons. The virus particles which were predominantly unenveloped occurred in the nucleus and more rarely in the cytoplasm (Fig. 8). Naked capsids were also occasionally seen in nuclei of monocytes. Virus was not seen in the perineurial epithelial cells of the perineurium and extracellular virus was not seen in any region of the nerve.

Spinal ganglion

At 84 h p.i. small numbers of virus capsids were observed in the nucleoplasm of some neurons and at all subsequent stages in neuronal nuclei and cytoplasm. Enveloped virus particles were frequently seen in the cytoplasm of infected neurons but particles were not observed extracellularly in the ganglion at any stage of the disease. Virus capsids were rarely seen in the nuclei of satellite cells and never in the cytoplasm and infected neurons were almost invariably surrounded by a sheath of uninfected satellite cells. As in the nerve, virus was occasionally seen in axons and Schwann cells within the ganglion.

DISCUSSION

The results of virus isolation and fluorescent, light and electron microscopic studies confirm and extend the findings of previous workers (Hurst, 1933; Sabin, 1937; Dow & McFerran, 1962, 1966; Becker, 1968). Virus multiplied in the fasciae at the inoculation site for 48 to 60 h and subsequently appeared almost simultaneously in the local nerve and related spinal ganglion and spinal cord segment. As the disease progressed virus spread cranially and caudally along the spinal cord from the initially infected site so that by death virus was present throughout the central nervous system. The findings are indicative of a centripetal spread of virus along the peripheral nerve to the central nervous system.

It has been postulated that herpes virus may gain access to the spinal ganglion and cord by being transported in the perineurial fluid, endoneurial fluid or axoplasm, or by replication and progression in Schwann cells, endoneurial connective tissue cells or perineural epithelial cells (Wright, 1953; Johnson & Mims, 1968). The findings, in the present study, are not compatible with a cellular pathway. In the nerve, virus was never detected in perineural epithelial cells and only occasionally in interstitial and Schwann cells. Further, there was no evidence that these cells were producing and releasing infective virus. The findings in the present study, albeit in a relatively small number of animals, are not compatible with a cellular pathway. Virus appeared almost simultaneously throughout the nerve, related spinal ganglion and cord segment between 60 and 84 h p.i. and there was no suggestion of a centripetal stepwise progression. Furthermore, since the replication cycle of pseudorabies virus takes a minimum of 5 h (McCracken & Clarke, 1971) it would be impossible for virus to spread progressively from cell to cell along the 75 cm of nerve in the time. Such rapid movement can only be achieved in a fluid or semi-fluid medium and the only such media available are the perineurial fluid, endoneurial fluid and axoplasm of nerve fibres.
Available evidence suggests that the perineurial fluid is an unlikely conduit for virus transport in the nerve. In the present study virus was not detected either in the perineural epithelial cells or in the surrounding tissue spaces and virus antigen was restricted to the endoneurium. Furthermore, as the perineurial fluid is continuous with the cerebrospinal fluid (Shantha & Bourne, 1968), transport of virus in the perineurial fluid would probably result in a generalized meningitis with virus being present in the cerebrospinal fluid. However, it has been demonstrated that a generalized meningitis does not occur and that virus is not present in the cerebrospinal fluid during the course of the disease (McFerran & Dow, 1964).

Fluid has been demonstrated to exist in the endoneurial spaces (Weiss et al., 1945; Steer & Horney, 1968) and it is possible that virus may travel in this fluid to the central nervous system. However, the findings of the present studies are not consistent with such a route. Virus was never observed in the extracellular spaces within the endoneurium and ganglion and was detected only in relatively small numbers of Schwann cells and very rarely in satellite cells. Infection of neurons almost invariably occurred without infection of surrounding satellite cells and it is therefore very unlikely that virus reached neuronal nuclei from the extracellular space of the ganglion. The absence of infection of satellite cells surrounding neuronophagic nodules containing mature virus particles suggests that these cells form an efficient barrier to virus movement. The random distribution and the absence of a hilar concentration of infected neurons in the ganglion are not indicative of endoneurial fluid transport. Steer (1971) has shown that there is a potential barrier to fluid movement between the peripheral nervous system and the central nervous system at their junction in the nerve roots and it could be anticipated that virus travelling in the endoneurial fluid would appear in the nerve and spinal ganglion before the related spinal cord segment. However, virus appeared in the central and peripheral nervous tissues simultaneously in the present studies.

The axoplasm was originally regarded as a virus conduit to the central nervous system (Goodpasture & Teague, 1923; Goodpasture, 1925) but this route subsequently lost favour when it was demonstrated that the axoplasm has a highly viscous consistency and streams slowly centrifugally at a rate of approximately 1 mm/day (Weiss & Hiscoe, 1948; Lubinska, 1964). However, it was later shown that some axoplasmic proteins are transported centrifugally at a rate exceeding that of the slow components by 100 to 500 times (Lasek, 1968; McEwen & Grafstein, 1968; Ochs, Sabri & Johnson, 1969); thus some proteins can travel up to 50 cm in the axons of mammalian nerves within 24 h. It has further been established that a centripetal flow of some amino acids and proteins occurs in mammalian axoplasm (Lasek, 1967; Watson, 1968; Kristensson & Olsson, 1971), but the rate of centripetal movement has yet to be ascertained. It is therefore conceivable that virus may be transported centripetally from a peripheral site to related neuronal nuclei in the spinal ganglia or cord.

In the present studies virus was first seen in ganglionic neurons at 84 h p.i. and by 120 h p.i. particles were present throughout the length of infected axons. The location and distribution of virus particles within the nerve indicate that they must have originated in the neuronal nucleus and subsequently travelled centrifugally up to 75 cm in the axoplasm in less than 36 h. No direct evidence of centripetal transport of virus was obtained in the early stages of the disease when virus uptake must have occurred. Since Morgan, Rose & Mednis (1968) have shown that the cellular uptake of herpesviruses results in only the core or capsid appearing in the cytoplasm, it is not surprising that the relatively crude techniques employed were unable to detect one or two virus particles within a nerve length of 75 cm. Final proof
of axoplasmic transport of infecting pseudorabies virus must await the employment of a technique capable of detecting single naked capsids.

Some recent work with the closely related virus, herpes simplex, lends support to the hypothesis of axoplasmic transport. Johnson (1964) originally suggested from fluorescent antibody and virus titration studies that virus travelled progressively along Schwann cells and fibroblasts and early ultrastructural studies supported this hypothesis (Rabin, Jensen & Melnick, 1968; Severin & White, 1968). However, as Wildy (1967) pointed out ‘it remained to be formally demonstrated whether the fluorescing cells really contributed to spread by producing infective virus or whether they were merely silent witnesses of centrifugally moving virus in the tissue spaces’.

More recently it has been shown that herpes simplex virus can be isolated from the spinal ganglion and cord of laboratory animals simultaneously or before it can be isolated from the related nerve (Baringer & Griffith, 1970; Kristensson, 1970a). From ultrastructural studies several workers have suggested the axoplasm as the possible virus conduit (Baringer & Griffith, 1970; Kristensson et al. 1971) and particles have been observed in the axoplasm of mice experimentally infected with herpes simplex virus (Hill, Field & Roome, 1972). Stevens, Nesburn & Cook (1972) have demonstrated that in latent herpes simplex infection in laboratory animals virus is harboured in the ganglion and not in the brain or peripheral nerve. This finding lends support to the hypothesis that recurrent herpetic infections are a result of centrifugal axoplasmic transport of virus from the sensory neurons to the cutaneous tissue. However, it has not been unequivocally established that the virus travels in the axoplasm and recently Rajcani & Conen (1972) concluded that virus moved centripetally in the endoneurial spaces rather than in the axoplasm. However these experiments were performed on suckling mice in which Kristensson (1970b) has shown that the endoneurium is not as yet completely isolated from the surrounding tissues. The immature nerve and its endoneurium is therefore more readily accessible to subcutaneously inoculated virus and results obtained may not be relevant to infection in older animals.

The excellent technical assistance of Mr S. P. Rodgers, Mr T. Connor and Mr W. Curran is gratefully acknowledged.

REFERENCES


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