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The clinical response of cattle experimentally infected with lumpy skin disease (Neethling) virus

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Summary. British cattle were inoculated with lumpy skin disease (Neethling) virus and their clinical signs observed over a three week period. Elevation of body temperature following infection was not found to be a consistent feature, and even in severe cases was limited to a peak temperature of 41 °C. Generalised lesions were seen 9–14 days post infection (p.i.), and the development of generalised infections did not appear to be dose related. Following intradermal inoculation lesions were detected from day 2 p.i. and first appearance and severity of local reaction appeared to be related to dose. Virus isolation was carried out on ocular, nasal and saliva swabs, and on buffy coat preparations. A transient viraemia was detected in two of eleven animals that did not show generalized signs; virus was not isolated from the secretions of seven animals without generalised signs. Virus was isolated from the peripheral secretions of an animal with generalised disease between 9 and 15 days p.i. and viraemia was detected in each of five animals with generalized signs. Delayed-type hypersensitivity reactions following intradermal inoculation of immune cattle with LSDV were found to be maximal at 24 h after challenge.

Introduction

Lumpy skin disease (LSD) is a severe pox disease of cattle restricted to Africa and the Middle East, and characterised by nodular skin lesions, lymphadenopathy and, in severe cases, death [5]. Production losses due to LSD may be large, due to hide damage [9], decreased milk yield and prolonged debility following infection [10]. There is strong circumstantial [1, 15, 24] and increasing experimental [2, 24] evidence that arthropod vectors are essential in the transmission of LSD. The importance of generalisation in predisposing to the rapid spread of other pox diseases by contact [12, 20] and by arthropods [7, 8] is well documented. Excretion of virus and environmental contamination or direct transmission is important for the transmission of many viral diseases of ruminants, and viraemia is a necessary prerequisite for mechanical transmission of certain other viruses by blood feeding diptera [14].

Analysis of clinical signs allows the determination of prognostic indicators, which could be of value for the control of disease spread in a herd situation, and the results of virus isolation from infected animals indicate the relative importance of animals showing local or generalised disease signs in the vector transmission of the virus. This paper describes the isolation of LSD virus in secretions and the blood of cattle infected by the inoculation of virus onto the conjunctiva or by the interadermal, intravenous or intranasal routes.

Materials and methods

Virus strains

A virulent South African Neethling strain of capripoxvirus (LSD-SA), originally recovered from a cow with LSD and then passaged in cattle at the Institute for Animal Health (IAH), Pirbright [13] was used as described below as the challenge virus for the experimental animals. Primary lamb testis (LT) cells were prepared from prepubertal lambs [17], and cultured in 175 cm^2 tissue culture flasks in Glasgow modified Eagle's medium supplemented with glutamine (GMEM) and 5% foetal calf serum. Lachrymal fluid from a heifer infected with LSDV, or biopsy material as a 10% suspension in phosphate buffered saline (PBS) was used to infect a 90% confluent cell culture. After 1 h at 37 °C the cell culture was washed with PBS and overlaid with GMEM. Virus was harvested when the characteristic cytopathic effect [17] was seen in 90% of the cells. The flask and contents were frozen at −20 °C, thawed, and the cell debris pelleted at 500 **g** for 20 minutes. The supernatant was removed, titrated as described below, and used for inoculation of experimental animals.

Virus titration

Lamb testis cells were seeded in 50 µl GMEM at 6×10^6 cells per ml in all wells of 96 well plates. Fifty µl of $\log_{10}$ dilutions of the virus suspension were used to infect rows A-G of the cells. Fifty µl of GMEM was added to row H, the cell controls. Cells were examined daily, from day five to day nine, for cytopathic effect [17]. The virus titre was calculated from the number of wells infected on day nine [11].

Experimental animals

Friesian-cross cattle aged 9 months to 4 years old were kept in the disease secure facilities at IAH, Pirbright. Whenever possible yearling heifers and bullocks were used, but older non-lactating cows were also used. Animals were inoculated onto the conjunctiva (n = 2), intranasally (n = 2), intradermally (n = 25) or intravenously (n = 11). Intradermal and intravenous inoculations were carried out as described previously [2] Inoculation of virus on to the conjunctiva and intransally was carried out in the xylazine-sedated (Rompun, Bayer) animal. Following intranasal instillation of virus the animal's head was kept raised for approximately three minutes. All animals received between 10^2 and $10^{6.5}$ $TCID_{50}$. Inoculation sites were examined daily for the first two weeks after inoculation, and thereafter at least once every three days. Clinical reaction was recorded on a scale 0–10 as described previously [2], with 1–6 indicating degrees of local response, and 7–10 degrees of generalisation (Table 1).

The body temperature of each animal was recorded daily. Blood samples were collected at intervals into heparinised tubes on ice for virus isolation. Ocular, nasal and throat swabs

Table 1. Scale of clinical response following infection of cattle with lumpy skin disease virus (Neethling)

Reaction score	Severity of clinical response
10	Severe generalisation leading to culling. Numerous secondary nodules, 0.5–5 cm diameter, with oedema, hyperaemia and pain. Severe lymphadenopathy, conjunctivitis, rhinitis, severe debility and inappetance.
9	Severe generalisation with depression. Numerous secondary nodules, severe lymphadenopathy, conjunctivitis and rhinitis.
8	Generalisation with many secondary lesions and severe lymphadenpathy. No systemic disturbance.
7	Generalisation with few secondary nodules, severe lymphadenopathy, no systemic disturbance.
6	Severe local reaction at inoculation site: heat, pain, oedema, lesion > 6 cm diameter. Severe lymphadenopathy.
5	Severe local reaction: heat, pain, oedema, lesion > 6 cm diameter. Prescapular lymph node twice normal size.
4	Local reaction moderately severe: > 6 cm diameter, some heat, pain and oedema. Mild lymphadenopathy.
3	Mild local reaction (< 6 cm diameter) and lymphadenopathy.
2	Mild local reaction: < 5 cm diameter. No lymphadenopathy.
1	Transient local reaction.
0	No detectable reaction.

were collected into sterile GMEM with antibiotics (sodium penicillin 10^3 iu/ml; streptomycin sulphate 5 mg/ml; mycostatin $10^{2.7}$ iu/ml; neomycin $10^{2.9}$ iu/ml).

Ten animals were challenged intradermally following recovery from a primary infection. 10^4–10^5 $TCID_{50}$ LSD-SA were inoculated intradermally into the skin of the neck, and the site of inoculation and local lymph nodes examined daily for three days, and thereafter at least once every three days.

Isolation of virus from swabs and blood samples

Virus isolation was performed on LT cells, in 25 cm^2 tissue culture flasks. Bijoux containing swabs collected from the experimental animals and 5 ml GMEM were incubated at 4 °C on a rotational shaker for 1 h prior to adsorption onto LT cells for 1 h at 37 °C. The cells were then washed and overlaid with GMEM. The flasks were inspected for cytopathic effect (CPE) daily for 12 days using the light microscope, and following freeze/thawing passaged onto fresh LT monolayers for a further 12 days.

Heparinised blood samples were kept on ice until and during processing. The samples were centrifuged at 2000 rpm for 15 min, and the buffy coat carefully removed into 5 ml of cold double distilled water, to lyse remaining erythrocytes. After 30 seconds 5 ml of cold X2 Glasgow modified Eagle's medium (GMEM) was added and mixed. The mixture was centrifuged at 2000 rpm for 15 min, the supernatant discarded and the cell pellet suspended in 5 ml GMEM. After further centrifugation for 15 min the resulting pellet was suspended in 5 ml of fresh GMEM. One ml of the cell suspension was adsorbed onto an LT cell monolayer in a 25 cm^2 flask for 45 min, before addition of 5 ml GMEM with 1%

foetal calf serum (FCS). Cells were examined daily for 12 days for cytopathic effect (CPE). If negative after 12 days, the cultures were frozen to −20 °C and thawed and clarified tissue culture supernatant used to inoculate fresh cells.

Results

Clinical observations

Body temperature

Rise in body temperature was not a consistent feature following infection. Animals which developed generalised lesions had body temperatures not exceeding 41 °C, and rises in temperature were usually observed more than one week post infection (p.i.), irrespective of route of inoculation (Fig. 1). In contrast,

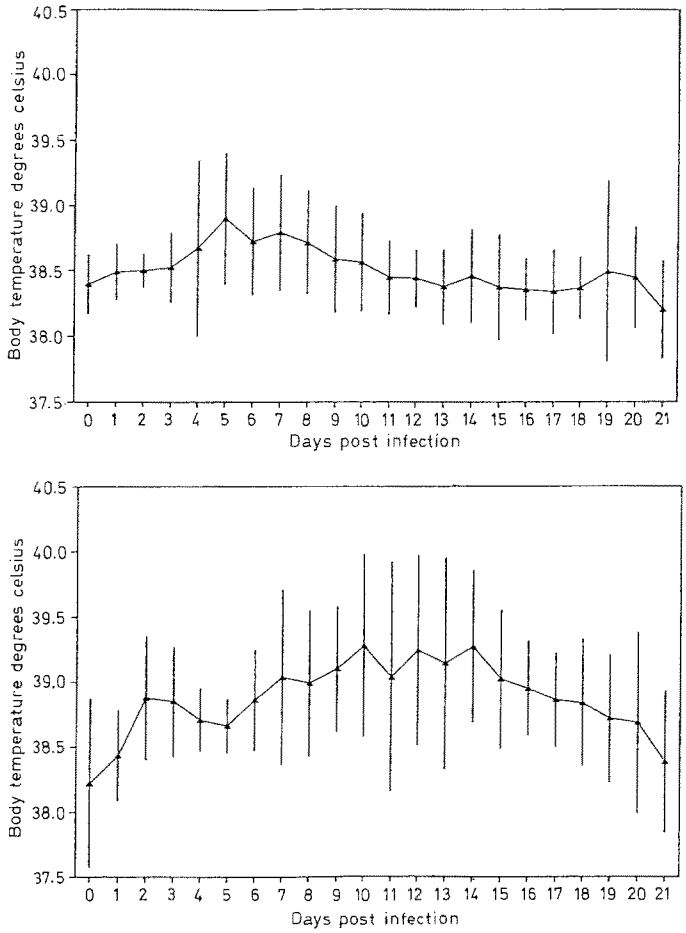

Fig. 1. Body temperature of cattle following infection with LSDV. Top graph: animals that developed local inoculation site lesions showed a transient temperature rise within the first week p.i. Bottom graph: animals that developed generalised lesions developed a temperature rise that lasted over one week p.i.

Table 2. The incubation period for secondary lesions and the clinical reaction score of cattle showing generalised lumpy skin disease following experimental inoculation with lumpy skin disease virus (Neethling) by different routes

Animal	Route of infection	Infecting dose $TCID_{50}$	Reaction score	Incubation period (days)
1	ID	6.7	9	7
2	IV	4.3	8	9
3	IV	4.3	8	9
4	IV	4.3	9	9
5	IV	5.3	8	9
6	IV	3.9	10	12
7	IV	5.3	9	12
8	IN	3.0	7	13
9	IV	3.3	7	13
10	IV	3.9	8	13
11	ID	6.0	10	13
12	ID	2.0	9	14
13	ID	6.0	9	14

ID Intradermal
IV Intravenous
IN Intranasal
$TCID_{50}$ Tissue culture infective dose

animals that subsequently developed only local inoculation site lesions showed transient temperature rises within the first week following inoculation (Fig. 1). Davies [4] reported no age or sex predisposition to the affects of LSD virus, and results here were not correlated with age or sex of experimental animal.

Incubation period

Time between inoculation and first observation of generalised clinical signs is given in Table 2. The incubation period ranged from 7 to 14 days, irrespective of the route of infection.

Dose response

High infecting doses were associated with early appearance of local lesions following intradermal challenge (Fig. 2). The titre of the infecting dose was also associated with severity of local lesion: animals that did not develop a generalised infection, and therefore received a reaction score of less than 7, were more likely to receive a high reaction score if they were inoculated with a dose of LSDV more than $10^{4.5}$ $TCID_{50}$ (Fig. 3). The severity of generalised disease in those animals that had response scores greater than 6 was not related to the titre of the infecting dose. Doses of 1 or 10 $TCID_{50}$ produced no local response in 2 animals, whereas both animals receiving doses of 100 $TCID_{50}$ developed generalised infection.

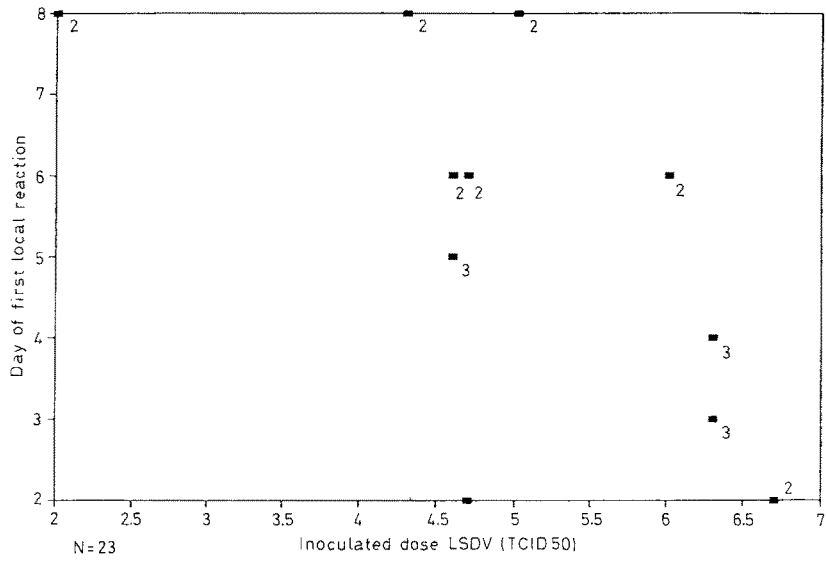

Fig. 2. Day of first observed local lesion in cattle inoculated intradermally with LSDV. High infecting doses were associated with the early appearance of local lesions following intradermal challenge

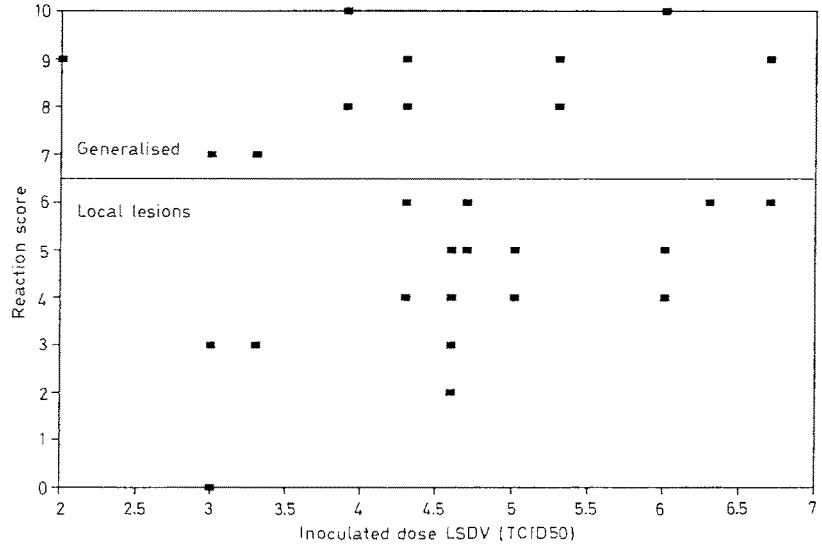

Fig. 3. Reaction score of cattle inoculated with different doses of LSDV. The severity of the local lesion in animals that did not develop generalised infection was directly related to the titre of the infecting dose. However, there was no relationship between infecting dose and development of a generalised infection

Delayed type hypersensitivity reaction

All ten animals showed maximal delayed-type hypersensitivity reaction (DTH) 24 h p.i. Local lymphadenopathy was observed at 24 h in 4 animals and at 48 h in 5 animals. The reaction persisted in the majority of animals for at least a week, and had disappeared by two weeks.

Virus isolation

Ocular, nasal and salivary swabs

Six animals with local intradermal inoculation site lesions were sampled regularly from day 4 p.i. to day 12 p.i. and on no occasion was virus isolated from the swabs after two tissue culture passages. One other animals with a local lesion was sampled daily up to day 15 p.i. and no virus was isolated from the secretions on any of these days. One animal with generalised disease was sampled daily following infection. Virus was isolated from ocular swabs on days 10, 12, 13, and 14, and from saliva on days 9 and 12.

Buffy coat

Eight animals, inoculated intradermally (n = 7) or intravenously (n = 1), which did not develop generalised disease, were consistently negative for virus isolation from the buffy coat, attempted either daily (in one case) or regularly between day 4 and 16. One animal inoculated intranasally was positive on day 6 p.i. and one of two animals inoculated onto the conjunctiva was also positive on day 6 p.i. Five animals which subsequently developed generalised lesions were sampled. Virus was isolated from each animal at least once between days 3 and 13 p.i. All animals were negative by day 16, and the earliest isolation was made on day 3, from an animal which had been inoculated intradermally. The results are shown in Table 3.

Discussion

Lumpy skin disease is characterised by generalised nodular skin lesions, although usually less than 50% of cattle experimentally infected with LSD virus or naturally exposed during an outbreak develop generalised infection [19, 24]. It has not been possible to show the transmission of LSD in the absence of an insect vector under experimental conditions, even when the donor animal has severe disease [2]. If, therefore, as field evidence has long suggested, an insect vector is required for transmission of LSD, the more sites on an infected animals that virus is available to infect or contaminate a feeding insect, the greater the possibility for transmission. The major insect vector of LSD virus has not yet been identified, but, considering the insect transmission of other pox viruses, transmission is probably mechanical, and a variety of insects are therefore potentially capable of transmitting virus. Reports of field outbreaks of LSD describe situations in which outbreaks are associated with severe and generalised

Table 3. Virus isolation from white blood cells of cattle inoculated with lumpy skin disease virus (Neethling)

Animal	Route of infection	Reaction score	Day of attempted virus isolation														
			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
14	I/CONJ	3				−		+			−				−			−
15	IN	3				−		+			−				−			−
16	I/CONJ	0				−		−			−				−			−
17–22	ID	6				−			−		−							
23	IV	6				−		−			−							−
24	ID	6	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	
8	IN	7				−		+			+				+			−
2	IV	8	−			−		−			+				+			−
3	IV	8	−			−		−			+				−			−
1	ID	9	−	−	+	+	+	+	+	+	+	+	+	−	−	−	−	
4	IV	9	−			−		+			+				+			−

I/CONJ Inoculation to the conjunctiva
IN Intranasal, *ID* intradermal, *IV* intravenous

infections and high mortality, and other instances in which there are few obviously infected animals or deaths [4]. It is possible that this reflects the involvement of strains of different pathogenicity, although Kitching et al. [13] were unable to show differences in LSD virus isolates collected over a 20 year period from various African countries using restriction endonuclease analysis. An alternative explanation, consistent with the variability of response seen in cattle experimentally infected by the intradermal route, is that the severity of disease in field outbreaks is related to the efficiency of transmission by the insect vector, and the method by which it feeds. Carn and Kitching [2] have previously reported that intravenous infection is more likely to produce generalised infection, and would therefore predispose to more severe outbreaks of disease.

There are no published reports of the experimental infection of such a large number of cattle from which sequential samples have been collected. Prozesky and Barnard [19] infected seven cattle by intradermal inoculation at 10 sites, and collected samples for virus isolation at autopsy. Only three of the seven cattle developed generalised infection, and in only two of these three was virus isolated from the blood. This study extends their results, and showed that two out of eleven cattle with local reactions developed a transient viraemia, whereas five out of five cattle with generalised infection developed a viraemia of longer duration, one of which lasted nine days (Table 3). Both animals infected intranasally became viraemic: one developed generalised disease and was viraemic for at least 8 days, the other showed no clinical signs and was viraemic for a maximum of 4 days. These results indicate the potential for infection via the nasal mucosa when sufficiently high titres (10^3 $TCID_{50}$) of virus are present.

The lack of virus transmission by contact [2] may reflect the relatively low levels of virus being excreted, although the insufflation of virus containing material, either saliva or scabs, during mutual grooming must remain as a possible route of infection in the field, but probably of little epidemiological importance.

No LSD virus was isolated from eye, nose or salivary swabs collected regularly from seven cattle which developed only local lesions at the site of inoculation. However, LSD virus was isolated from ocular and saliva swabs collected from an animal with generalised infection. Many species of *Muscidae* feed around the eyes and mouths of cattle and feeding behaviour is likely to be enhanced by the presence of increased and mucopurulent material associated with disease. Cattle with generalised LSD would be a potent source of virus for insects feeding around the head, and if these were vectors of LSD would necessarily predispose to rapid spread of disease. Interrupted feeding behaviour is a regular event for many *Muscidae*, and the observation that one of two animals inoculated with LSDV onto the conjunctiva developed a transient conjunctivitis and viraemia indicates that this route of infection may be important in the field, and that the sponge-like feeding apparatus and regurgitation during feeding of *Muscidae* may have relevance to the transmission of virus.

Further work is required to determine the minimum infective dose of LSDV by different routes, but the results presented in this paper indicate that doses in excess of 10^1 $TCID_{50}$ are required to establish infection when inoculated intradermally, that 10^2 $TCID_{50}$ is sufficient to produce generalised infection by this route, and that 10^3 and $10^{3.3}$ $TCID_{50}$ may produce generalised disease by the intranasal and intravenous routes, respectively. This is of particular importance in the mechanical transmission of virus, when contamination of the insect is a highly variable occurrence. The low titre of LSDV shown in the blood of viraemic animals indicates that it would be unlikely for mechanical transmission to occur following feeding on blood alone. There is a requirement for mosquitoes to feed on the lesions of rabbits infected with myxoma virus in order for transmission to occur [7] and it is likely that the mechanical transmission of LSDV will be similarly constrained. High titres (10^4–$10^{8.2}$ $TCID_{50}$) of virus are found in skin lesions in both sheep pox [18] and LSD [23], which may be sufficient for contamination of biting insects mouthparts.

Although infecting dose was not related to degree of severity of disease observed in the animals in this study, it did correlate with the speed of development and the severity of the local lesion. This finding is in agreement with observations on the primary vaccinial lesion, made by Von Pirquet [22]. In those animals challenged intradermally a second time, the development of the maximal DTH reaction to live virus was consistent with that described by Capstick and Coakley [3] following the inoculation of inactivated LSD virus. Capstick and Coakley [3] found 77.5% (31/40) of animals immune to LSD produced a DTH reaction on inoculation of homologous, inactivated virus, whereas in this study, using live virus, 100% (10/10) reacted. This finding suggests that soluble viral antigens were not as effective as live virus at producing a DTH

reaction, and therefore that both CD4+ and CD8+ T lymphocytes are likely to be important in the initiation of the DTH response following infection.

Following primary infection the body temperature of those animals that subsequently developed generalised disease increased later than that of those that developed local lesions. Poxviruses are known to be temperature sensitive in culture, and in both mousepox [21] and myxomatosis [16] low environmental temperature greatly increased the severity of clinical disease. Although there is no recorded effect of body temperature in cases of smallpox, strains of variola minor virus, having a naturally low ceiling temperature, produced many fewer virions from infected cells when the temperature was raised [6], thereby diminishing dissemination. An early temperature rise in the LSDV infected animal may therefore provide some protection from generalisation of disease.

It is clear that by providing a variety of virus-contaminated feeding sites the animal with generalised infection is key to the transmission of LSDV. Further work is required to investigate the ability of African insects to transmit LSDV, and it is likely that certain vectors are more efficient than others and would predispose to further generalised infections. It would be important to identify these vectors, not only to assist in their specific control in order to reduce the spread of disease, but also to determine whether vectors capable to transmitting LSDV exist outside Africa.

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