Studies on the pathogenesis of rinderpest in experimental cattle

IV. Proliferation of the virus following contact infection

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Rinderpest can be transmitted easily to experimental cattle by many parenteral routes, but the natural method of infection remains unknown. Many text-books still state that infection takes place by the oral route in the ordinary course of events (Hutrya, Marek & Manninger, 1946; Henning, 1949; Davies, 1955; Blood & Henderson, 1960; Hagan & Bruner, 1961). This is in spite of evidence that drenching with large quantities of highly infectious material may fail to set up the disease (Schein & Jacotot, 1925; Hornby, 1926; Maurer, 1965) and, on the contrary, a great deal of information indicating the ease with which the virus invades the body when introduced via the upper respiratory tract (see Hornby, 1926; Hall, 1933; Maurer, 1965; Liess & Plowright, 1964; Plowright, 1964) or as an aerosol (Provost, 1958).

So far as we are aware, no systematic studies have hitherto been undertaken to demonstrate the route or routes by which virus invades the body in natural cases of rinderpest. Cattle were, therefore, killed at varying time intervals after controlled exposure to contact infection with a single virulent strain of virus and the infectivity in various tissues was determined by a tissue-culture technique. Particular attention was paid to parts of the upper and lower respiratory tracts, which previous investigations had indicated might provide a primary portal of entry. The results of this experiment were, in many respects, comparable to those obtained when cattle were infected by the intranasal instillation of virus (Plowright, 1964).

MATERIALS AND METHODS

Virus

The virulent RGK/1 strain of rinderpest (Liess & Plowright, 1964) was used throughout the experiment. At the time of its use the virus had undergone, in succession, two passages in primary calf kidney cells, one passage in cattle, an additional passage in primary calf kidney cells and, finally, three further passages in cattle. The animal utilized for the final passage (ox no. 9519) was killed on the morning of the 4th day of pyrexia, and portions of its spleen were stored at -70°C .

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Experimental animals and their infection

All experimental cattle were grade steers or heifers, aged between 1 and 2 years and either Ayrshire or Friesian crosses. Their sera contained no rinderpest-neutralizing antibodies (Plowright & Ferris, 1961).

The animals were housed in an isolation unit, where rigid precautions were taken to prevent transfer of infection from stall to stall. Hay was fed from the floor and water was available from a single trough in each stall; hence, opportunities for the dissemination of virus in any one stall were very good.

To produce cases of contact infection a series of animals was injected parenterally and used as virus donors. Experimental infections were produced by the subcutaneous inoculation of 2 ml. of a 10^{-1} or 10^{-2} suspension of the spleen of ox no. 9519 in culture maintenance medium; such inocula contained approximately $10^{4.8}$ or $10^{5.8}$ TCD₅₀ of virus and always gave rise to pyrexia on the 3rd to 5th morning following inoculation.

It was shown previously that rinderpest-infected cattle excreted virus in their nasal secretions, urine and faeces, but quantitative data suggested that the nasal excretions constituted a major source of contagion for other animals. Further, the percentage of infected animals excreting virus by the nasal route reached a maximum on the 4th day of pyrexia and high virus titres were encountered in the nasal secretions between the 3rd and 7th days of fever (Liess & Plowright, 1964). Accordingly, groups of two or three susceptible animals were introduced to stalls containing a virus donor, which had attained the 3rd, 4th or 5th morning of its reaction. After 24 hr. exposure the susceptible animals were transferred to separate clean stalls. To avoid the possibility of any later infection, the donor animals were immediately removed from the unit and the stall used for contact exposure was disinfected.

Collection of materials

Cattle were killed and exsanguinated at daily intervals from the 2nd to the 10th mornings after their first contact with a virus donor. Blood was collected in every case for viraemia estimation (Plowright, 1964) and for separation of serum.

The technique for collection of solid tissues was as already described (Plowright, 1964). The *pharyngeal mucosa* was obtained, in all except four instances, by removing a 2–3 cm. section along the median fold of mucous membrane, at a point lying immediately ventral to the insertion of the ventral straight muscles of the neck; the median fold here comprises several smaller, longitudinal ridges lying in close apposition to each other and extremely rich in lymphoid tissue.

Unless otherwise stated the following tissues were always collected: *Nasal mucosa* was obtained from the middle third of the dorsal turbinate bone. The *upper tracheal mucosa* was dissected from the ventral aspect of the organ, over the first three cartilaginous rings, and the *lower tracheal mucosa* from between the opening of the right apical bronchus and the bifurcation. The *left diaphragmatic bronchial mucosa* was obtained by opening the main branch of the left bronchus along its entire length; the mucosa could then be removed in strips of up to 4 cm.

in length, by plucking at it with rat-toothed forceps. The mucosa of the *tongue* was removed from the under surface of the free anterior part of the organ, over an area where the epithelium is tightly bound.

One each of the following *lymph nodes* was excised intact: *submaxillary*, *pharyngeal*, *left prescapular*, *left costocervical*, *left bronchial* and *left prefemoral*. Except in one instance a single large, or several small, *middle cervical lymph nodes* were collected, from either side of the trachea, in the middle of the neck.

A portion of *spleen* and an entire *palatal tonsil* were removed, while *lung* tissue was also obtained from the diaphragmatic surface of the *left diaphragmatic lobe* and from the hilar region of the *left cardiac lobe*.

Subsequent treatment of tissues

Where necessary, specimens were dissected to remove capsular and trabecular connective tissue. The technique used in the preparation of tenfold dilutions of solid tissue has already been described; so, also, have the preparation of blood leucocyte fractions and blood dilution series (Plowright, 1964).

Each preparation was inoculated in 2 ml. amounts into each of five tubes of primary calf kidney (BK) cells. It was found previously (Plowright, 1964; Taylor & Plowright, 1965) that, for solid tissues, dilutions lower than 10^{-2} could produce cytotoxic changes in BK cells and hence this was the highest concentration inoculated.

It soon became apparent that many animals would be tested in which no virus would be detected. Further, it was not possible to anticipate from the day of slaughter the amount of virus that would be present in positive animals. Consequently, after some initial trials, dilutions were chosen which ranged from 10^{-2} to 10^{-4} or 10^{-5} for solid tissues, and from 10^{-1} to 10^{-2} or 10^{-3} for whole blood, with the invariable inclusion of a leucocyte fraction. This procedure inevitably resulted in a failure to reach titration end-points in cases where virus generalization was well established, but for the present purpose such animals were of reduced significance.

Preparation and maintenance of cell cultures

The production, maintenance and post-inoculation treatment of BK cultures was as already detailed. Final microscopic observations were made on the 9th day, except in the case of the tissues of ox no. 9927, when bacterial contamination made it necessary to terminate readings on the 7th day. Titres were calculated as before (Plowright, 1964).

Serum neutralization tests

Sera collected at the time of slaughter were screened for rinderpest-neutralizing antibodies by the method of Plowright & Ferris (1961); the test dose of virus was $10^{1.6}$ TCD₅₀ per tube.

RESULTS

Clinical and post-mortem observations

Two animals (nos. 9771 and 9776) were killed on the first morning of pyrexia; in both of these virus was recovered in large amounts from many tissues (see

Tables 1 and 2). No fever or other clinical abnormalities were observed in the remaining cattle exposed to contact infection. Lymph node congestion was noted in two animals (nos. 9997 and 9931), in both of which virus had become generalized.

Virus proliferation

A total of 35 animals were investigated and virus was detected in only 15 of them. The distribution of virus in the tissues of these 15 animals is given quantitatively in Tables 1 and 2.

Table 1. *The titre of virus in the tissues of cattle killed on days 3-6 following contact exposure to rinderpest*

Time after exposure ...	3 days		4 days		5 days	6 days			
Animal no. ...	9927*	9999	9975	9997	9925	9762	9766	9771	9931
Nasal mucosa	0.0	0.0	0.0	0.0	0.0	0.0	2.4	0.0	0.0
Pharyngeal mucosa	0.0	0.0	0.0	≥ 4.2	3.8	0.0	≥ 6.2	6.7	4.6
Upper tracheal mucosa	0.0	0.0	0.0	1.8	4.4	0.0	≥ 4.2	6.4	2.4
Lower tracheal mucosa	0.0	0.0	0.0	2.4	2.6	0.0	≥ 4.2	6.0	2.7
Bronchial mucosa	0.0	0.0	0.0	1.8	2.2	0.0	≥ 4.2	4.6	3.0
Lung—hilar	0.0	0.0	0.0	Tr†	0.0	0.0	≥ 4.0	3.0	1.6
Lung—diaphragmatic	0.0	0.0	0.0	Tr	0.0	0.0	≥ 4.2	3.8	Tr
Pharyngeal lymph node	2.2‡	3.4	1.6	≥ 4.2	3.8	2.4	≥ 6.2	6.6	4.4
Mid-cervical lymph node	0.0	0.0	0.0	≥ 4.2	3.4	1.7	≥ 5.2	6.0	2.4
Costocervical lymph node	0.0	0.0	0.0	3.4	≥ 5.0	3.2	≥ 6.2	6.2	2.8
Bronchial lymph node	0.0	0.0	3.0	3.8	4.4	0.0	≥ 6.2	6.7	3.6
Tongue mucosa	0.0	0.0	0.0	0.0	0.0	0.0	1.6	1.6	0.0
Submaxillary lymph node	0.0	0.0	0.0	3.8	4.0	2.6	≥ 6.2	6.4	5.0
Tonsil	2.8	0.0	0.0	≥ 4.2	≥ 5.2	2.6	≥ 6.2	7.6	3.4
Blood	0.0	0.0	0.0	0.7	1.2	0.4	≥ 2.2	2.6	1.4
Spleen	0.0	0.0	Tr	≥ 4.2	4.2	2.4	≥ 6.2	6.2	2.8
Prescapular lymph node	0.0	0.0	0.0	≥ 4.0	3.6	1.6	≥ 6.2	6.2	3.4
Prefemoral lymph node	0.0	0.0	0.0	3.4	3.4	0.0	≥ 6.2	6.2	0.0

* Observations for ox no. 9927 terminated on day 7 owing to bacterial contamination of cultures.

† Tr = Trace, i.e. one tube was infected of five inoculated with 10^{-2} dilution of solid tissue.

‡ Titre expressed as $\log_{10}$ TCD 50/g., or per ml. (blood).

Whereas no virus was detected in one animal killed on the second day following exposure, two of five destroyed on day 3 did show virus multiplication. Of these, no. 9927 had moderate amounts of virus in both the tonsil and pharyngeal lymph node, whereas in no. 9999 virus was found only in the latter (Table 1).

On day 4 virus was recovered from two of the six cattle tested. One of them (no. 9975) showed virus in the pharyngeal and bronchial lymph nodes, while a low-grade viraemia must also have occurred, as traces of virus were recovered from the spleen. In the other animal that was positive at this time, no. 9997, the

virus had become generalized, as evidenced by its presence in the blood, pre-scapular and prefemoral lymph nodes, etc.

Of the six animals killed on day 5, infectivity was demonstrated in the tissues of one only (no. 9925) and in this individual virus had also generalized. On day 6, virus was recovered from four of the six cattle tested. In three, nos. 9766, 9771 and 9931, the virus was widely disseminated; no. 9762 showed viral multiplication in the pharyngeal, mid-cervical, costocervical and submaxillary lymph nodes; also in the tonsil, blood, spleen and prescapular lymph node.

Table 2. *The titre of virus in the tissues of cattle killed on days 7-10 following contact exposure to rinderpest*

Time after exposure ...	7 days			8 days		10 days
Animal no. ...	9760	9779	9870	9776	9781	9757
Nasal mucosa	0.0	2.0	0.0	1.8	0.0	0.0
Pharyngeal mucosa	N.T.	≥ 4.2	≥ 4.2	N.T.	N.T.	N.T.
Upper tracheal mucosa	0.0	≥ 2.2	1.6	≥ 3.2	0.0	0.0
Lower tracheal mucosa	0.0	≥ 2.2	2.2	≥ 3.2	0.0	0.0
Bronchial mucosa	0.0	≥ 2.2	≥ 3.2	≥ 3.2	0.0	0.0
Lung—hilar	0.0	≥ 3.2	0.0	≥ 4.2	0.0	0.0
Lung—diaphragmatic	0.0	≥ 3.2	2.0	≥ 4.2	0.0	0.0
Pharyngeal lymph node	2.4*	≥ 5.2	≥ 5.2	≥ 5.2	2.2	2.4
Mid-cervical lymph node	Tr†	≥ 4.2	4.0	≥ 5.2	1.8	0.0
Costocervical lymph node	0.0	≥ 4.2	2.8	≥ 5.2	N.T.	0.0
Bronchial lymph node	0.0	≥ 5.2	4.8	≥ 6.2	3.8	0.0
Tongue mucosa	0.0	1.8	0.0	0.0	0.0	0.0
Submaxillary lymph node	0.0	≥ 5.2	4.4	≥ 5.2	2.0	2.0
Tonsil	3.2	≥ 5.2	4.4	≥ 5.2	1.8	0.0
Blood	0.0	≥ 3.0	Tr	2.6	Tr	Tr
Spleen	1.6	≥ 5.2	3.8	≥ 5.2	Tr	1.6
Prescapular lymph node	0.0	≥ 4.2	2.4	≥ 5.2	0.0	0.0
Prefemoral lymph node	0.0	≥ 4.2	3.2	≥ 4.2	0.0	0.0

* Titre expressed as $\log_{10}$ TCD 50/g., or per ml. (blood).

† Tr = Trace, i.e. one tube was infected of five inoculated with a 10^{-2} dilution of solid tissue, or by a blood leucocyte fraction.

N.T. = Not tested.

Four animals were killed on day 7, of which three yielded virus; in two, nos. 9779 and 9870, generalization had occurred, while in the third (no. 9760) virus was present in the tonsil, pharyngeal and mid-cervical lymph nodes. The recovery of virus from the spleen of this animal indicated a low-grade viraemia.

On day 8 the tissues of four cattle were harvested and in two instances virus was demonstrable. Generalization had occurred in no. 9776, but in no. 9781 virus was restricted to the pharyngeal, submaxillary, mid-cervical and bronchial lymph nodes, the tonsil, blood and spleen (Table 2).

No virus was recovered from either of the two animals killed on day 9, whereas no. 9757, sacrificed on day 10, had virus in its pharyngeal and submaxillary lymph nodes, blood and spleen.

Serum neutralization

As expected, no rinderpest-neutralizing antibody was detectable in the post-exposure sera of any of the experimental animals.

DISCUSSION

It was more difficult than expected to produce regular cases of rinderpest in cattle by 24 hr. contact exposure to single donor animals in the 3rd–5th days of the disease reaction. The infection in the recipients had sometimes become generalized in as little as 4 days, whereas in other instances no virus could be detected in selected tissues after 9 days. Liess & Plowright (1964), using the same strain of virus, at approximately the same passage level, noted regular contact infection after periods of 8–11 days with a mean of 8·6 days for 11 animals; in these cases the intensity of exposure during the first 2 days of pyrexia in the donor(s) was probably low or even negligible, increasing with the development of the disease in the donor(s).

The quantity of virus escaping in the nasal or other excretions of infected cattle may vary considerably from animal to animal. Thus, Liess & Plowright (1964) failed to demonstrate virus at any time in the nasal secretions of two out of 24 cattle, whilst urinary and faecal excretion was even more irregular. Such observations may well account for the fact that five donor cattle in these experiments failed to produce demonstrable infection in either of the two animals which were housed with them and killed 2–6 days later; they would also explain the great variation in the rapidity of development of the infection in the present experiments, assuming that the length of the eclipse phase depends on the dose of virus received.

A comparable irregularity of contact transmission with some Indian strains of rinderpest virus was reported by Cooper (1932), who found that exposure periods of 10 days sometimes failed to convey the infection to susceptible cattle, while other animals in continuous contact with presumed virus excretors did not react for 31–33 days.

Among the 15 animals from which virus was recovered, 13, unfortunately, had a viraemia at the time of sampling or must already have circulated virus, since it was demonstrable in the spleen. However, in five of these cattle dissemination was limited, as shown by the failure to obtain virus from the mucosae of the respiratory tract, lung parenchyma and prefemoral lymph node. Titration data for these five and two positive animals killed on day 3 are assembled in Table 3 and will serve as a basis for the following discussion.

The pharyngeal lymph node was invariably involved and this receives lymph from the tongue, floor of the mouth, hard and soft palates and gums, all covered by stratified squamous epithelium and thus unlikely to represent the primary portal of virus entry—at least when uninjured. Also draining to the pharyngeal lymph node are the mucosae of the pharynx and posterior part of the nasal cavity, the maxillary and palatine sinuses and the larynx (Sisson & Grossman, 1953).

The submaxillary lymph node was infected in three instances, the titre of virus being low and comparable in each case to that in the pharyngeal node of the same animals; this node receives afferent vessels from the muzzle, lips, cheeks, gums, hard palate and tip of the tongue—all provided with stratified squamous epithelium—the anterior parts of the turbinate bones and septum nasi. The evidence from the two cephalic nodes, even in the absence of virus proliferation in the mucosae, gives support to the hypothesis that part, at least, of the infecting virus entered through the nasal mucosae or, less probably, the associated sinuses and pharynx.

Table 3. *Results for animals which gave indications of the route(s) of infection*

Time after exposure ...	3 days		4 days	6 days	7 days	8 days	10 days
Animal no. ...	9927*	9999	9975	9762	9760	9781	9757
Nasal mucosa	—	—	—	—	—	—	—
Pharyngeal mucosa	—	—	—	—	N.T.	N.T.	N.T.
Upper tracheal mucosa	—	—	—	—	—	—	—
Lower tracheal mucosa	—	—	—	—	—	—	—
Bronchial mucosa	—	—	—	—	—	—	—
Lung—hilar	—	—	—	—	—	—	—
Lung—diaphragmatic	—	—	—	—	—	—	—
Pharyngeal lymph node	2·2	3·4	1·6	2·4	2·4	2·2	2·4
Mid-cervical lymph node	—	—	—	1·7	Tr†	1·8	—
Costocervical lymph node	—	—	—	3·2	—	N.T.	—
Bronchial lymph node	—	—	3·0	—	—	3·8	—
Tongue mucosa	—	—	—	—	—	—	—
Submaxillary lymph node	—	—	—	2·6	—	2·0	2·0
Tonsil	2·8	—	—	2·6	3·2	1·8	—
Blood	—	—	—	0·4	—	Tr	Tr
Spleen	—	—	Tr	2·4	1·6	Tr	1·6
Prescapular lymph node	—	—	—	1·6	—	—	—
Prefemoral lymph node	—	—	—	—	—	—	—
Probable route of infection	UR	UR	LR UR(?)	LR UR(?)	UR	LR UR	UR

Titre expressed as $\log_{10}$ TCD 50/g., or per ml. (blood).

* Observations for ox no. 9927 terminated on day 7, owing to bacterial contamination.

N.T. = Not tested.

† Tr = Trace.

— = virus not detected in 0·1 g. tissue or leucocytes from 13 ml. blood.

UR = upper respiratory tract. LR = lower respiratory tract.

Four animals showed very early proliferation of virus in the palatal tonsil, which drains only its own epithelium, all of stratified squamous type and lining the crypts. It is interesting to note that two cattle which showed virus in the tonsil (nos. 9927 and 9762) had no detectable infection of the pharyngeal mucosa, which has a comparable histological structure of stratified squamous epithelium, closely associated with nodular lymphoid tissue. In discussing the vulnerability of the tonsil to microbial infections, Payling Wright (1954) observed that the epithelium lining the depths of the tonsillar crypts presents certain structural weaknesses, in the form of thinning or even actual defects (Stöhr's lacunae).

He also admitted that it was not known whether leucocytes could wander out into the crypts, engulf pathogenic organisms and return to the tissue of the tonsil. Either explanation could account for this structure being a primary site of proliferation of rinderpest virus.

In two cases (nos. 9975 and 9781) the highest titre was recorded in the bronchial lymph node, which contained more than 20 times as much virus as any other structure. The spleen showed evidence of early generalization in each of these animals but it seems reasonable to suppose that the lung was the site of entry for virus which passed to the local lymph node, without undergoing detectable multiplication in either the bronchial mucosa or the lung parenchyma. In ox no. 9762, the greatest quantity of virus was found in the left costocervical lymph node. Again, there was evidence of early generalization and an absence of virus from the associated tracheal and bronchial mucosae; nevertheless, the indications were that infection had occurred through these surfaces. The occurrence of virus in the mid-cervical nodes of three of the animals in Table 3 may not have implied primary multiplication there, since the titre was very low in each case and in the same animals extension to the spleen had already occurred, presumably following a low-grade viraemia.

The results presented in Table 3, admittedly small in numbers, would support the hypothesis that rinderpest infection is naturally acquired via the mucosae of the upper and also, in some instances, via the lower respiratory tract. It is of interest to observe that the experimental susceptibility of the lower respiratory tract was reported by Hornby (1926), who infected cattle by the intratracheal inoculation of infectious material. Primary proliferation cannot be demonstrated at the presumed sites of mucosal penetration but virus is rapidly transported to the local lymph node and either multiplies there or passes very quickly through into the circulation, giving rise to a low-grade viraemia and localization in the spleen or other lymphopoietic structures. This theory is also supported by the data of Plowright (1964), who could not demonstrate primary proliferation of virus in the nasal mucosae of animals infected by intranasal instillation.

Some of the moderately high titres found in these experiments for virus in the pharyngeal, tracheal and bronchial mucosae of cattle which were still in the incubation period of the disease (see, for example, nos. 9766, 9997 and 9925) offer a possible explanation for the finding that the nasal excretions may occasionally become infective as early as the 2nd day preceding pyrexia (Liess & Plowright, 1964). It does not follow, of course, that high virus content in a mucosal tissue will necessarily imply excretion from the relevant surface; this has already been shown to be a false assumption in poxvirus infection of the respiratory tract of the rabbit (Bedson & Duckworth, 1963). It is also noteworthy that, whereas, in cattle infected by intranasal instillation, virus was not detected in the nasal mucosae until the 2nd day of fever, three of 13 animals in these experiments (nos. 9766, 9779, 9776) did have small quantities of virus in this situation before the onset of pyrexia.

So far as we are aware, the only comparable experimental study of naturally acquired cases of a viral exanthem is that of Bedson & Duckworth (1963), who

investigated rabbit pox. They encountered similar difficulties in infecting all their animals, since 12 of 31 did not yield virus at the time of sampling. However, in their experiments there was a tendency for rabbits killed later after exposure to show a wider dissemination and higher titres of virus than those killed earlier; this, surprisingly, was not our experience with naturally acquired rinderpest. Bedson & Duckworth (1963) encountered cases of both upper and lower respiratory tract infection, but in many instances a complete 'primary complex' was established, with virus proliferation both at the presumed surface of entry and in the regional lymph node.

SUMMARY

Cattle were infected with rinderpest virus by housing them for 24 hr. in stalls containing donor animals which had been reacting to the disease for 3-5 days. They were then transferred to individual clean stalls and killed on the 2nd to 10th days following first exposure. Various tissues were collected, particularly those of the upper and lower respiratory tracts, and their virus content was estimated in calf-kidney tissue cultures.

Virus was recovered from 15 of 35 animals tested and in eight of these generalization had occurred, although only two had begun to show a pyrexial response. The stage of the infection could not be predicted from the time that had elapsed following exposure, since early, limited proliferation was encountered on the 3rd to the 10th days.

It was considered that seven animals gave indications of the pathways by which natural infection had occurred. In each of these virus proliferation was established very early in the pharyngeal lymph node; in three the submaxillary lymph node was similarly involved and in four the palatal tonsil. It was suggested that these data probably indicated that infection always occurred via the upper respiratory tract.

In three cases virus titres were highest in the bronchial or costocervical lymph nodes; this was construed as evidence for the additional involvement of the lower respiratory tract in primary infection.

No infectivity could be demonstrated in the mucosae or lung parenchyma associated with the above-mentioned lymph nodes and this, together with previously published data, was accepted as strong presumptive evidence that the infecting virus passes through the mucosae without producing a local lesion or proliferating there. These results were compared briefly with those of Bedson & Duckworth (1963) for rabbit pox.

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