Vol. XLIII, No. 4 (August, 1962), was issued on 14.8.62.

THE BRITISH JOURNAL OF
EXPERIMENTAL PATHOLOGY

VOL. XLIII OCTOBER, 1962 NO. 5

HISTOPATHOGENESIS OF MOUSEPOX
I. RESPIRATORY INFECTION

J. A. ROBERTS

From the Department of Microbiology, The John Curtin School of Medical Research, The Australian National University, Canberra, Australia

Received for publication January 26, 1962

INFECTION by inhalation has long been suspected as an important means of transmission of a number of poxviruses. Recently Hahon and Wilson (1960) have studied the infection of *Macaca irus* monkeys with airborne variola virus using infectivity assays. However, histological studies of early growth and dissemination have not been reported.

Edward, Elford and Laidlaw (1943) demonstrated that mice can be infected by airborne infectious ectromelia virus (mousepox). The present report describes the pathogenesis of mousepox acquired by airborne virus, studied by a combination of infectivity titrations and fluorescent antibody staining for the localisation of antigen in tissues.

MATERIALS AND METHODS

Virus.—The Hampstead Mouse strain of ectromelia, described by Fenner (1949), was used. Second passage chorioallantoic membrane material as a 10 per cent suspension in gelatine saline (0·5 per cent gelatine in borate buffered saline) was prepared as stock and stored in aliquots at −60°. One preparation was used throughout. The titre in $\log_{10}$ units/0·05 ml. was: chorioallantoic membrane inoculation 6·3; subcutaneous foot pad injection ID_{50} 6·9; intraperitoneal injection ID_{50} (= LD_{50}) 7·0; intranasal (0·05 ml.) inoculation LD_{50} 6·7.

Mice.—Outbred multicoloured mice of a strain derived from the Walter and Eliza Hall Institute, Melbourne, strain were used.

Aerosols.—An all-glass nebulizer manufactured by Burroughs Wellcome and Co. (Australia) Ltd. was used to produce aerosols. The pressure source is a rubber hand-bulb which directs the air vertically into the reservoir. The outlet at the top of the reservoir is a tube 0·8 cm. diameter and 3 cm. long at a right angle to the air jet. There is no buffer, so that a wide range of particle sizes is produced. Mice were exposed in groups of eight in boxes 28 × 15 × 13 cm., 250 ml. of aerosol being introduced at each of times 0, 15, and 30 min. After 45 min.

the mice were transferred to clean boxes. Whole lung titrations performed immediately after aerosol inoculation showed that 40–60 ID_{50} of virus were retained.

Sampling of organs for infectivity assays.—Mice were killed by bleeding from the axilla under ether anaesthesia and entire organs were aseptically dissected. Organ suspensions from individual mice were made and titrated separately.

Large parenchymatous organs such as liver, spleen and lungs were twice freeze-ground in a chilled pestle and mortar, and suspended in 2 ml. of gelatine saline ; bony tissues were fragmented with scissors and blended with 10 ml. of gelatine saline in 20 ml. containers with a Nelco homogenizer. The suspensions were then ultrasonically dispersed and the supernate titrated after centrifuging at 1200 *g* for 10 min.

Small organs in which it was desired to detect small amounts of infectious virus such as lymph nodes, Peyer's patches and trachea were twice freeze-ground in a chilled pestle and mortar, diluted with 1 ml. of gelatine saline and ultrasonically dispersed all in the same small tubes whence they could be titrated directly.

Infectivity assays.—The intraperitoneal inoculation of mice was selected as the titration system for two reasons. Firstly because up to 0·2 ml. of crude tissue suspension can be inoculated by this route and secondly because one infectious dose of a virulent strain of ectromelia kills mice (Schell, 1960). Four mice were inoculated with each 10-fold dilution and observed for 14 days. Fifty per cent end points were then calculated by the method of Reed and Muench (1938) and expressed as $\log_{10}$ units per organ.

Fluorescence microscopy.—Tissues were frozen in containers in a bath of liquid air and stored at −20°. Lymph nodes and other small tissues were mounted in 10 per cent gelatin in distilled water in a No. 4 gelatin capsule (Parke, Davis and Company, Sydney) before freezing. It was found that lungs would not section well if stored longer than 2 weeks.

A Cambridge rocker microtome in a −20° refrigerator was used to cut sections 6 μ thick and the sections were picked up by thawing them onto a glass slide (Louis, 1957). The sections were then dried under a fan at room temperature for at least 30 min., fixed for 10 min. in acetone, dried, and either stored at −20° or stained immediately.

The direct staining method was used, the γ-globulin being prepared from the serum of vaccinia hyperimmune rabbits and conjugated with fluorescein isothiocyanate (Sylvana Chemical Co., Orange, New Jersey, U.S.A.). The conjugate was adsorbed for 30 min. at 37° with each of acetone extracted mouse and rabbit liver powders, and then with live chick embryo fibroblasts. The adsorbed conjugate was stored at −20° until it was required, when it was diluted with two volumes of phosphate buffered saline, and 0·2 ml. of rhodamine conjugated bovine albumin (Microbiological Associates, Bethesda, Maryland) added to each ml. of diluted conjugate as a counterstain. The specificity of staining was tested by staining normal tissues with the antivaccinial conjugate. A control normal rabbit serum was prepared in the same way and representative sections from each experimental tissue were stained with it to detect non-specific staining.

Sections were overlain with the conjugate at room temperature in a moist chamber for 15 min. and then given three 10-min. washes in phosphate buffered saline before mounting in neutral glycerol. Sections were examined with a Zeiss microscope equipped for fluorescence observations and illuminated with an Osram HBO 200 high-pressure mercury lamp.

Two types of non-specific staining were seen in lungs. Occasional small stellate cells randomly distributed in the tissue exhibited complete cytoplasmic fluorescence, the number of such cells varying from 0 to 30–40 per $mm.^2$ field. With experience it was possible to differentiate them from infected cells ; however, if the control stain showed any of these cells all sections from the lung were discarded. The second type was seen as a halo of fluorescent cells and tissue around blood vessels and bronchioles in the lung. It was never possible to completely eliminate this, but adsorption of the conjugate with live chick fibroblasts reduced it to a pale green easily differentiated from a specific stain. In the upper respiratory tract a few mucus secreting glands in the submucosa exhibit a bright or pale green fluorescence. This can be easily differentiated because it is flocculant and not quite in the plane of the tissue when compared with the specific staining of a virus infected cell. It would be possible for this last type of non-specific fluorescence to obscure specific fluorescence.

If it was desirable to identify a particular cell the coverslip was floated off in 10 per cent formol saline after recording the fluorescence in a photomicrograph on Ilford HP_3 35 mm. film. The section could then be stained with haematoxylin and eosin. The resulting sections often made it possible to identify a cell and determine its type. However, the fine cytoplasmic and nuclear structure of individual cells could not be distinguished.

EXPERIMENTAL RESULTS

Lymphatic drainage of the respiratory system

Before commencing a study of infection in the respiratory tract it was necessary to determine the locations of lymph nodes and the areas drained by each node.

Two groups of 10 mice were injected by the intravenous and intraperitoneal routes with 0·5 ml. of a dilution of indian ink in normal saline. The mice were carefully dissected 3 months later to determine the location of the carbon labelled lymph nodes. The pulmonary nodes are illustrated in Fig. 1. Any of the nodes may be a doublet.

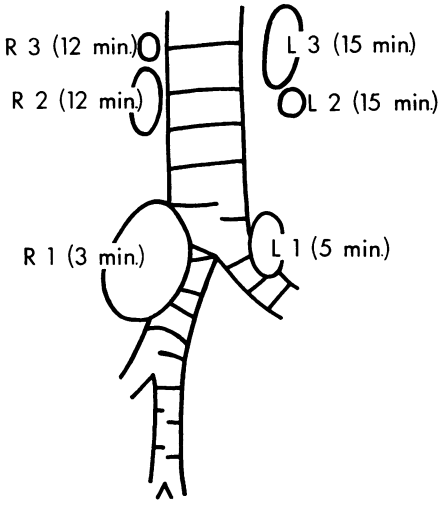


Fig. 1.—Lymph nodes included in the pulmonary lymph node pool.

The drainage from the respiratory tract was determined by inoculating mice under light ether anaesthesia intranasally with 0·05 ml. of 0·5 per cent Evans blue in normal saline and killing them at 1 min. intervals. Fig. 1 shows the time of appearance of the dye in the pulmonary lymph nodes. Most of the lymphatic drainage as shown by the intensity of staining was through the pulmonary nodes of the right side.

The pulmonary lymph node pool consisted of all the nodes illustrated in Fig. 1. The upper respiratory tract lymph node pool comprised all lymph nodes in the head and neck.

Virus growth in the mouse following aerosol infection with ectromelia virus

To determine the sites of primary infection and the sequence of the subsequent infection of organs, titrations were performed.

Mice were infected by aerosol, 4 mice were killed immediately, and then 4 more each day for 10 days. Pooled organs were titrated. The results are recorded in Table I and indicate that primary lesions occur in the head and lungs. Regional lymph nodes are not infected until the 3rd day, and the spleen is also infected by this time. Regional lymph nodes may be infected earlier when ectromelia is

TABLE I.—*Results of Daily Titrations of pools of Organs from Four Mice Infected with Ectromelia by Aerosol. Titres are expressed as* $Log_{10}ID_{50}$*/Organ*

Organ	Day 0	1	2	3	4	5	6	7	8	9	10
Lung	1·0	3·4	4·1	4·3	5·1	5·6	5·6	6·2	6·2	6·8	7·8
Pulmonary lymph nodes	0	0	0	1·3	2·1	4·5	5·8	6·1	4·1	3·8	7·1
Spleen	—	0	0	2·0	3·6	4·5	6·1	8·7	9·0	8·3	8·9
Head	—	3·1	4·2	5·0	5·2	—	—	—	—	—	—
Brain	—	0	0	0	0	—	1·2	—	4·1	—	—

Popliteal and inguinal lymph nodes, mesenteric lymph nodes and Peyer's patches tested on days 1–4 revealed no virus.

injected into the foot pad, Fenner (1948), a difference that may be attributed to the relatively atraumatic aerosol infection method. Deaths usually first occurred on day 7, and most mice died on days 8 and 9. The rises in organ titres over the last 2 days of the disease probably indicates a breakdown in antibody production due to destruction of lymphoid tissue by the virus.

In order to study further the spread of infection through the regional lymph nodes of the lung and the upper respiratory tract the nodes from individual mice

EXPLANATION OF PLATES

(Fluorescent antibody stained sections from mice infected by aerosol with ectromelia virus)

FIG. 2.—Eight hours after infection. A primarily infected alveolar macrophage in a section of lung. ×430.

FIG. 3.—Three days after infection. Respiratory bronchiole with focus of infected mucosal cells. In this section and the serial sections of the whole focus the infection had not penetrated the basement membrane (arrows). ×350.

FIG. 4.—Three days after infection. Respiratory bronchiole with focus of infected mucosal cells. Lymphatic endothelial cells (arrows) adjacent to the basement membrane of the respiratory bronchiole (right) and its associated artery (left) are infected. ×500.

FIG. 5.—Eight days after infection. Infected bronchiole (lower left). The lymphatic vessels about the artery (centre) are dilated and the endothelium is infected (arrows). There are infected macrophages and infected cell debris in the lumen of the lymphatic. The adjacent alveolae are not infected. ×140.

FIG. 6.—Eight days after infection. Infected region adjacent to a small artery in a section of lung. Infected macrophages (arrows) and infected cell debris are present in the dilated perivascular lymphatics. The vascular endothelium at the top right of the artery is infected. ×430.

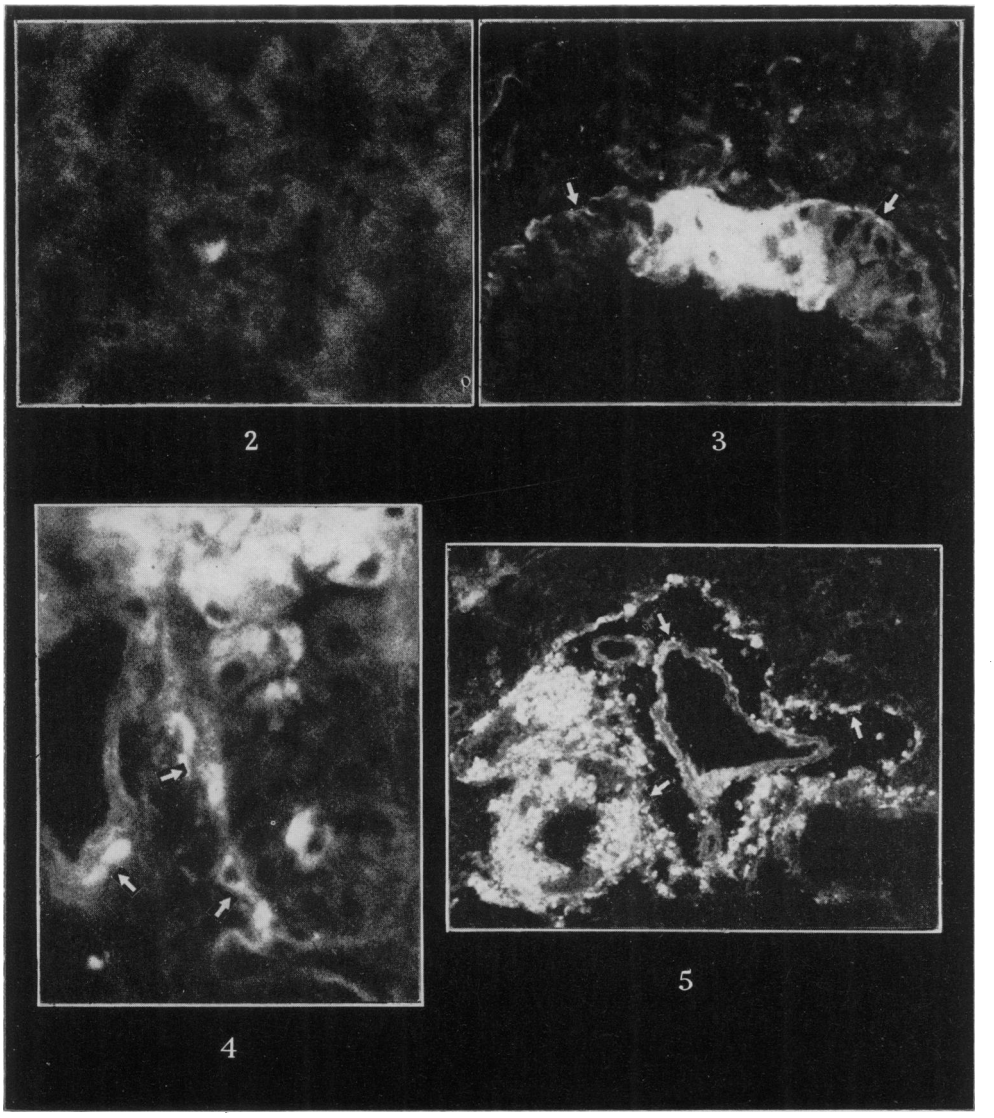
FIG. 7.—Three days after infection. Infected macrophage in a section of the pulmonary lymph node. ×500.

FIG. 8.—Four days after infection. Groups of fluorescent cells in a pulmonary lymph node. Most of the infected cells are macrophages some of which have infected adjacent lymphocytes. ×110.

FIG. 9.—Five days after infection. Dilated subcapsular sinus and medullary sinuses (arrows) in a pulmonary lymph node. Most cells around the margin of the node are infected but the region adjacent to the hilus (left) is unaffected. ×35.

FIG. 10.—Three days after infection. Infected olfactory mucosa (arrows) in section of the ethmoturbinate. The ethmoturbinate bone appears as a ribbon of non-specific fluorescence across the plate $\frac{1}{3}$ of the distance from the bottom. Just above the ethmoturbinate bone and to the right of the infected olfactory mucosa are infected macrophages and submucosal cells. ×55.

FIG. 11.—Six days after infection. Infected perineurium and endoneurium in a longitudinal section of olfactory nerve. The infection has spread from primarily infected olfactory mucosa (beyond the left-hand margin of the figure) and is in cells within the cribriform plate of the ethmoid bone (right-hand margin of the figure). ×430.

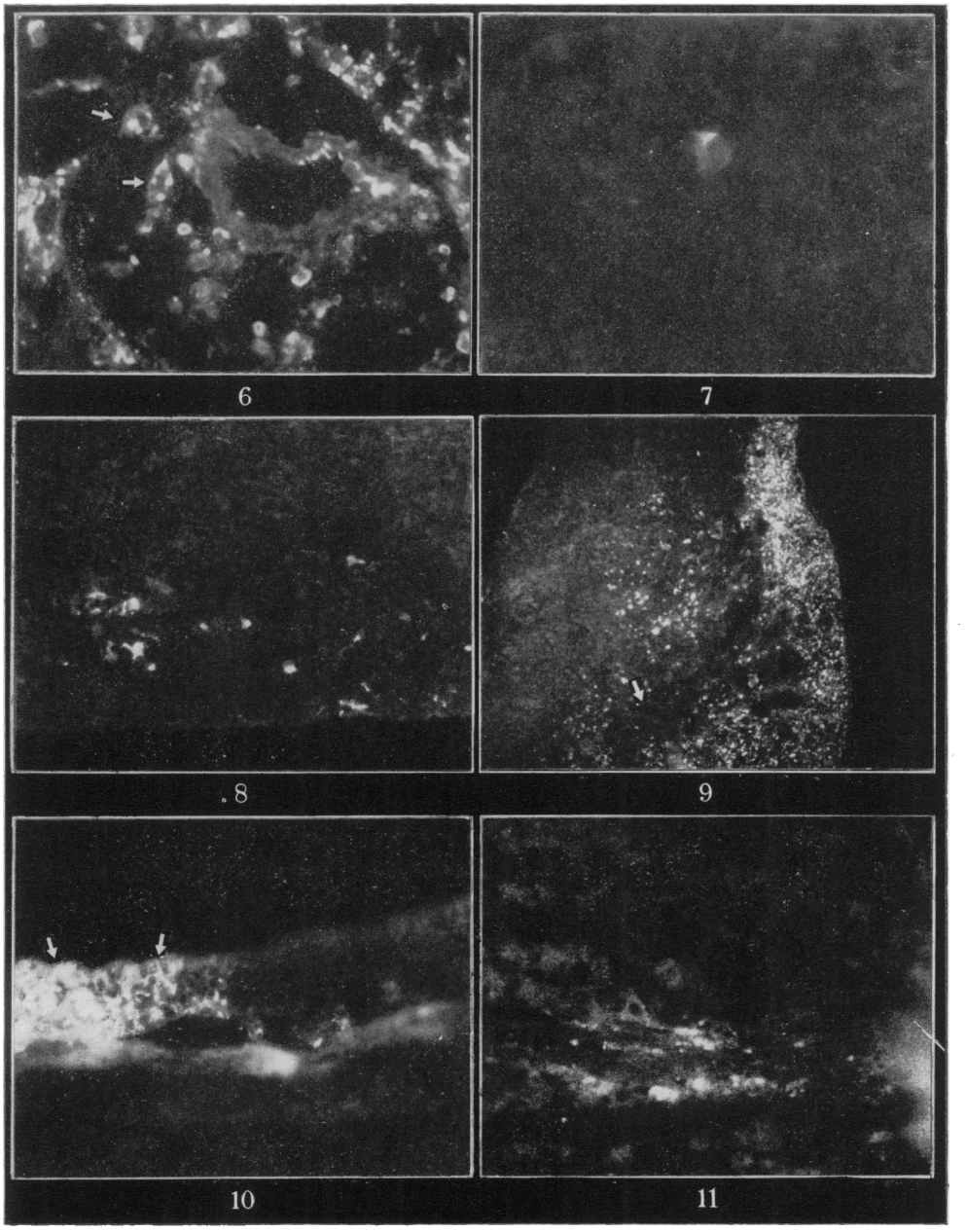


2

3

4

5



6 7

8 9

10 11

TABLE II.—*Results of Titrations of Lymph Node pools of the Upper and Lower Respiratory Tracts of Individual Mice Infected with Ectromelia by Aerosol. Titres are expressed as $log_{10}ID_{50}$/pool. Spleen Infection Indicates Spread of Virus from the Respiratory Tract to the Viscera*

	Day	Pulmonary lymph nodes Mouse				Cervical lymph nodes Mouse				Spleen Mouse			
		A	B	C	D	A	B	C	D	A	B	C	D
Exp. I	1	0	0	0	0	0	0	0	0	0	0	0	0
	2	0	0	0	0	0	0	0	0	0	0	0	0
	3	0	0·7	1·3	0	1·0	1·9	0	0·7	+	+	+	+
Exp. II	1	0	0	0	0	0	0	0	0	0	0	0	0
	2	0	0	0	0	0	0	1·4	0	0	0	0	0
	3	1·4	2·6	1·0	0	3·6	1·5	2·0	2·8	+	+	+	+

+ Virus was present but was not titrated.

were titrated in 2 experiments. Table II gives the results of the titrations. In each individual both the spleen and either the pulmonary or upper respiratory tract nodes were infected at 3 days. Although lymph nodes did not contain infective virus until the third day (Table II), it was thought possible that they nevertheless contained soluble antigen at an earlier stage. Passive cutaneous anaphylaxis, micro tube precipitation and gel diffusion techniques, however, failed to detect antigen at 2 days.

Fluorescent antibody studies of lung sections

In the above experiments virus growth was detected in the lung one day after aerosol infection. To obtain a full picture of the pathogenesis of infection 4 entire lungs were serially sectioned and stained at intervals up to 72 hr., and thereafter representative blocks of serial sections were examined. The first lungs examined had been infected for 8 hr. at which time no more than one growth cycle can have occurred. The observations were supplemented by the examination of formalin fixed, paraffin embedded, haematoxylin and eosin stained sections.

Specific fluorescence shows as one or more bright yellow inclusion bodies in the cytoplasm of a cell in which the rest of the cytoplasm stains faintly yellow green. This is in contrast to an uninfected cell which takes up the orange-red of the rhodamine counter-stain.

Eight hr. after infection.—The first virus growth occurred in either a single mucosal cell of a small bronchiole, or an alveolar macrophage. Alveolar macrophages can be recognized by their appearance after haematoxylin and eosin staining, and by the fact that they are always spread out and free, not distorted as would be expected if they were septal cells or located in the interalveolar partition. A primarily infected alveolar macrophage is illustrated in Fig. 2. In the complete lungs of 4 mice examined at 8 hr. the infected cells were distributed as follows :

Mouse 1	3	macrophages	2	mucosal	cells
,, 2	2	,,	2	,,	,,
,, 3	0	,,	0	,,	,,
,, 4	2	,,	0	,,	,,

One day after infection.—The number of fluorescent alveolar macrophages detected remained unchanged, but the number of mucosal foci, which were now 2–3 cells in diameter, was increased. Probably more viral particles have gained access to mucosal cells by this time.

Two days after infection.—The mucosal foci have spread by infection of adjacent mucosal cells but remain strictly demarcated from the rest of the lung tissue at the basement membrane. The number of fluorescent macrophages was greatly increased, there being about 200 such cells to each lung.

Three days after infection.—The mucosal foci had expanded further and most were still contained by the basement membrane (Fig. 3). Occasionally cells around the bronchiole or its associated artery contained antigen (Fig. 4). These infected cells were believed to be endothelial cells of the lymphatic channels (Miller, 1937). The pleura may show infection at this time, and rarely a small focus of infected alveolar septal cells was seen. Infected macrophages were common.

Four days after infection.—Most infected bronchioles and their associated artery now had fluorescent lymphatic endothelium around them. Sometimes an artery not associated with an infected brochiole also had a fluorescent lymphatic associated with it. The lumen of the lymphatic vessel occasionally contained an infected macrophage.

Six days after infection —The lymphatic channels were dilated and contained infected macrophages. A few large groups of alveolae and areas of pleura were involved. There was fluorescent mucosal debris in the lumen of bronchi and bronchioles. Small areas of alveolae were fluid filled and there was a general infiltration of monocytes and polymorphs. Polymorphs were never observed to be infected.

Eight days after infection —The lymphatic vessels were grossly dilated and contained much cellular debris and many fluorescent macrophages (see Figs. 5 and 6). Occasionally endothelial cells of large vessels contained antigen (Fig. 6), and the walls of the large veins were sometimes infiltrated by infected cells. There were extensive areas of consolidation with fluid and cellular infiltration.

Fluorescent antibody staining of sections of the pulmonary lumph nodes

The manner in which virus reaches the pulmonary lymph nodes and the location of virus after growth in the node was determined. Pools of the large lymph node, R1 in Fig. 1, from groups of 10–12 mice were serially sectioned in entirety 3 days after aerosol infection, and representative blocks of serial sections taken at later times. Specific fluorescence showed brightly against a pale background and no non-specific fluorescence was observed.

Very occasional infected macrophages were detected at 3 days (Fig. 7), the time at which virus was first detected in the titration experiments (Table II). Many nodes contained no antigen. At 4 days there were single and small groups of fluorescent cells usually in the cortical region of the lymph node (Fig. 8). These groups of infected cells progressively enlarged and increased in number until by 8 days the lymph node was sometimes completely destroyed. Often in the late stages there was still an area of lymphatic tissue close to the hilum of the node little affected by the infection. At six days when lymphatic dilation occurred in the lung the sinusoids of the lymph nodes were also dilated (Fig. 9).

As the first antigen in the pulmonary lymph nodes is in free macrophages and

not in the fixed phagocytic cells lining the sinuses of the node, it is probable that the infected macrophages have migrated from the lung.

Natural airborne virus

Dried superficial lesions and carcases when stirred up by the activities of mice in a cage are an obvious source of airborne virus. No respiratory activity comparable to coughing or sneezing was ever observed among infected mice, so it was of interest to determine whether infective virus could be expelled naturally from the respiratory tract.

Ten mice were infected by aerosol and were changed to clean boxes daily. The mice were prepared by clipping the vibrissae from the head and laying the hair back with a disinfecting solution of iodine-alcohol-glycerol. Each day the nose of each mouse was allowed to protrude through a hole in the end of a tube. The tube was suspended for 10 min. vertically above the dropped chorioallantoic membrane of an 11-day incubated fertile hen egg with the nose about 2 cm. above the membrane. Streptomycin and penicillin (100 units each) in 0·1 ml. of saline were then placed on the membranes and the eggs were sealed and incubated for 3 days. The membranes were often obscured by non-specific lesions but up to 20 pocks were counted on some membranes. The membranes were then ground and suspended in gelatine saline before being inoculated into mice to detect virus. The results are recorded in Table III.

TABLE III.—*Infection of the Chorioallantoic Membrane by Expulsion of Virus from the Respiratory Tract of Mice Infected with Ectromelia by Aerosol.*

	Mouse									
Day	1	2	3	4	5	6	7	8	9	10
1	—	—	N	N	—	N	—	N	—	N
2	N	N	N	N	—	N	—	—	—	+
3	N	—	—	—	N	N	—	—	N	N
4	N	—	—	—	—	—	—	+	—	N
5	+	—	—	—	N	—	N	+	N	—
6	N	+	+	+	N	+	N	+	+	+
7	..	..	+	..	+	+	+	+	..	..

\+ indicates the membrane contained virus after incubation.
— indicates the membrane did not contain virus after incubation.
N indicates the embryo died during incubation of the egg.

The titration experiments in Table I and III indicate that the virus expelled by mice on days 2 and 4 must have come from the respiratory tract, rather than from the mouth. Mice infected by the aerosol method described did not survive long enough to have a secondary exanthem and since all mice expel virus by day 6 it is apparent that virus may be expelled to the environment in the pre-exanthem period.

Fluorescent antibody studies of the upper respiratory tract

As a preliminary to microscopic studies, titrations were done of the head and neck region of aerosol infected mice. The result (Table IV) indicated that the ethmoturbinates, maxillary sinus, and nasal mucosa merited histological study.

TABLE IV.—*Results of Titrations of a Breakdown of the Heads of Individual Mice Infected with Ectromelia by Aerosol. Titres are expressed as* $log_{10}ID_{50}$*/Organ*

Anatomical region	Day 1				Day 2				Day 3				Day 4			
	Mouse				Mouse				Mouse				Mouse			
	A	B	C	D	E	F	G	H	I	J	K	L	M	N	P	Q
Maxillary sinus	0·3	0	1·0	0·7	2·1	1·8	2·9	2·0	3·4	2·0	2·9	2·9	5·1	4·2	4·3	3·1
Ethmoturbinate	2·0	1·3	1·3	1·8	3·7	2·6	3·1	3·5	4·3	3·1	4·7	5·2	4·3	2·9	3·4	3·7
Mucus membrane and olfactory mucosa of nasal turbinates and septum	0·3	1·2	0·7	1·0	3·0	1·7	2·7	2·2	3·3	2·7	2·9	4·5	3·1	4·1	5·6	3·7
Cervical lymph nodes	0	0	0	0	0	0	1·4	0	3·6	1·5	2·0	2·8	4·1	5·7	3·8	3·8
*Rest	0	0·7	1·3	0	2·0	0·9	2·9	1·1	2·7	4·1	1·3	2·2	2·3	5·2	5·2	4·6

* The rest consisted of bone, frontal sinus and small areas of mucosa of nasal, buccal and pharyngeal cavities. Mouth and tongue, pharynx, larynx, trachea, eye and conjunctiva, middle and internal ear, salivary glands, interscapular gland, and brain were also tested on each day. The only positive results were : Day 2, Mouse E, Pharynx (0·3) ; Day 3, Mouse J, Middle and internal ear (2·3) ; Day 4, Mouse N, Trachea (0·3).

Accordingly an examination was made of complete serial sections of these areas from groups of 4–12 mice 3 days after infection by aerosol.

Histological examination showed that about half the mucosa covering the nasal turbinates and septum was olfactory mucosa and that it was here that most of the antigen was located. There were, however, 1 or 2 small mucosal foci in the nasal turbinates and septum and in rare cases scattered cells in the associated glandular tissue. In the maxillary sinus there were rare mucosal lesions similar to those in the nasal mucosa and isolated infected macrophages deep in the submucosal glandular tissue.

There were about 3 or 4 lesions in the olfactory mucosa of the ethmoturbinates of each mouse as well as about the same number of lesions in the olfactory mucosa of other regions. These lesions were up to 20 cells in diameter involving the sustentacular cells of the mucosa and the full thickness of 3–12 olfactory sensory cells (Fig 10). Where submucosal and glandular tissue was present there were scattered small areas of submucosal cells associated with the lesion (Fig. 10).

Because of the direct connection of the olfactory neurones with the olfactory bulb of the brain this pathway was examined at 6 days when the brain is infected (Table I). The head of a mouse infected by aerosol 6 days earlier was carefully dissected away to leave the anterior cerebrum, olfactory bulb, cribriform plate, ethmoturbinates and medial nasal septum. The whole of this was mounted in gelatine and complete longitudinal serial sections taken and stained with fluorescent antibody. About 20 per cent of several hundred sections and parts of sections were mutilated by pieces of bone carried by the knife ; however, by the use of serial sections a clear picture of the distribution of antigen emerged. Three large areas of olfactory mucosa and submucosal tissue were infected. As the olfactory nerve increased in size away from the mucosa, the perineurium and endoneurium could be seen to be infected, the antigen forming a sheath about the nerve and being in the form of occasional streaks in the body of the nerve, often associated with a nucleus (Fig. 11). The particular cells involved could not be

identified but Schwann cells, macrophages and fibroblasts were all present. Continuity of antigen was followed through the cribriform plate into the area superficial to the glomeruli of the olfactory bulb, where again it was often associated with a nucleus probably of a glial cell. There was no orientation of antigen towards glomeruli where the olfactory axons synapse. The distance traversed by the infection in this manner from the olfactory mucosa varied from 0·5–3·5 mm. and each of the 3 mucosal foci was connected to a focus in the olfactory bulb. No other antigen was present in the olfactory bulb.

Sections of a pool of olfactory bulbs at 8 days showed that antigen is still contained in the area superficial to the glomeruli but sometimes involved small areas of the meninges also.

DISCUSSION

Alveolar macrophages have been demonstrated with the electron microscope by Karrer (1958) as a constant feature of young mouse lungs, where they usually occur adjacent to an alveolar septum and make contact with the epithelial lining by means of small pseudopodia. The careful studies of Ross (1957) in which guinea-pigs were exposed to spore clouds of *Bacillus subtilis* clearly implicated alveolar macrophages in the ingestion and transport of spores to the regional lymph node. The present experiments show that after aerosol infection, ectromelia virus is taken up by alveolar macrophages and that it grows in at least some of them. Infection may also be initiated in a mucosal cell of a respiratory bronchiole, or in the upper respiratory tract. Ross (1957) differentiated between phagocytosis by septal cells and by free alveolar macrophages, and while Karrer (1958) reports limited phagocytosis by the alveolar epithelium, no evidence was found in the present study for growth of ectromelia in the septum of the alveolus until the third day. Gogolak (1953) studying the aerosol infection of mouse lung with murine pneumonitis virus found septal cells infected in the first cycle of growth but no infected macrophage was found until the second cycle at 44 hr.

In the experiments described above, virus might have spread from the lung to the viscera by growing in the capillary endothelium and alveolar epithelium because infection of the alveolar septa first occurs at 3 days. However, pulmonary lymph nodes were also infected by the 3rd day and it was at this time that the spleen was first infected. It seems certain that one route of virus spread from the lung to the viscera is by the movement of infected macrophages through the pulmonary lymph node, for these cells were found in the node at 3 days when whole node titres are low. In the experiments of Yoffey and Sullivan (1939) where vaccinia virus was introduced onto the nasal mucosa of rabbits, virus could be detected at 12 hr. both in the cervical lymph node and in association with cells in the cervical lymph. Lymphatic drainage of the lung is via the network of lymphatics about the bronchioles and blood vessels and *via* the lymphatics of the pleural surface. Both these sites become infected at 3 days.

The perivascular and peribronchial lymphatics later become more severely infected and from 6 days become dilated, as do the sinuses of the pulmonary lymph nodes. Antibody would be produced at this time and thus while the fluid may result from some form of lymphatic blockade, it more probably indicates an inflammatory or allergic reaction. Miller (1937) has described microscopic collections of lymphatic tissue in mouse lung where alveolar macrophages loaded with dust particles may accumulate. In spite of the fact that serial sections were examined

there was no evidence of involvement of such lymphatic nodules either in the lung or the submucosa of the upper respiratory tract.

It is noteworthy that most of the primary mucosal lesions in infected mice were in nonciliated nonkeratinized epithelia of the respiratory bronchioles and the olfactory epithelium, while lesions were uncommon in the nasal mucosa, sinuses, pharynx, larynx, trachea, bronchi and conjunctiva. That most of the mucosal lesions do not penetrate the basement membrane for 3 days is interesting when compared with the olfactory mucosa where the absence of basement membrane is associated with advanced infection of the olfactory sensory cells at three days. It is, however, possible that the olfactory sensory cells are infected through their receptor processes at the surface. Burnet and Lush (1936) reported growth of ectromelia in the olfactory mucosa of the rat and spread of the virus to the olfactory bulb of the brain. The present studies show that in the mouse, ectromelia grows in the sustentacular and sensory cells of the olfactory mucosa and spreads to the olfactory bulb by growth in the cells of the perineurium and endoneurium of the olfactory nerves. Schwann cells, fibroblasts and macrophages are all present in the supporting tissues of the nerve and infection may travel both by cell to cell spread and by migration of infected cells.

MacCallum, McPherson and Johnstone (1950) and Downie (1951) have speculated about the site of primary infection of smallpox in man and about the fact that some patients are infectious in the pre-exanthem period. This study throws some light upon these problems. In natural infection any one of the types of primary lesion described could occur. A primary lesion in the mucus membrane of a nasal sinus, in the olfactory epithelium, or in a respiratory bronchiole could be overlooked at autopsy or discounted as a secondary lesion. Indeed, theoretically, an infected alveolar macrophage could migrate through the lymphatics and there need be no primary lesion at the time of autopsy. It has also been shown that in the incubation period of mousepox, virus may be expelled into the environment from the respiratory tract of mice infected by aerosol.

SUMMARY

Immune fluorescence has been used to analyse respiratory infection of mice with ectromelia virus.

The first infected cells are either alveolar macrophages or mucosal cells of the upper or lower respiratory tract.

Virus is not detectable in the regional lymph nodes until the 3rd day after infection and is then located in free macrophages.

The outstanding feature of the pulmonary lesion from 3 days is growth of virus in the lymphatic endothelium. After 6 days when antibody production has begun there is gross dilation of pulmonary lymphatics and the sinuses of the hilar lymph node.

Spread of virus from the olfactory mucosa of mice infected by aerosol *via* the perineurium of the olfactory nerve to the olfactory bulb of the brain is described.

Aerosol infected mice expel virus from the respiratory tract during the pre-exanthem period.

Helpful discussion with Dr. C. A. Mims during the experimental work and preparation of the paper is gratefully acknowledged.

REFERENCES

BURNET, F. M. AND LUSH, D.—(1936) *J. Path. Bact.*, **42**, 469.
DOWNIE, A. W.—(1951) *Lancet*, **260**, 419.
EDWARD, D. G. FF., ELFORD, W. J. AND LAIDLAW, P.—(1943) *J. Hyg., Camb.*, **43**, 1.
FENNER, F. J.—(1948) *J. Path. Bact.*, **60**, 529.—(1949) *Aust. J. exp. Biol. med. Sci.*, **27**, 31.
GOGOLAK, F. M.—(1953) *J. infect. Dis.*, **92**, 254.
HAHON, J. AND WILSON, B. J.—(1960) *Amer. J. Hyg.*, **71**, 69.
KARRER, H. E.—(1958) *J. Biophys. Biochem. Cytol.*, **4**, 693.
LOUIS, C. J.—(1957) *Stain Tech.*, **32**, 279.
MACCALLUM, F. O., MCPHERSON, C. A. AND JOHNSTONE, D. F.—(1950) *Lancet*, 259, 514.
MILLER, W. S.—(1937) ' The Lung '. Springfield, Ill. (Thomas).
REED, L. J. AND MUENCH, H.—(1938) *Amer. J. Hyg.*, **27**, 493.
ROSS, J. M.—(1957) *J. Path. Bact.*, **73**, 485.
SCHELL, K.—(1960) *Aust. J. exp. Biol. med. Sci.*, **38**, 289.
YOFFEY, J. M. AND SULLIVAN, E. R.—(1939) *J. exp. Med.*, **69**, 133.