WEST NILE VIRUS INFECTION IN MICE FOLLOWING EXPOSURE TO A VIRAL AEROSOL

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MANY of the laboratory infections acquired by people working with arboviruses seem to point to an airborne route of infection. Routine laboratory procedures such as are used in the preparation and handling of dried antigens or the accidental formation of a virus aerosol have been considered to be the sources of some infections in laboratory workers (von Magnus, 1950 ; Haymaker, Sather and Hammon, 1955). The contaminated dust in infected animal boxes, suspected by some investigators to be associated with other cases of laboratory infections, appeared to involve the respiratory route of infection (Lennette and Koprowski, 1943 ; Morse, Russ, Needy and Buescher, 1962). In a previous communication (Nir, 1959), a case of West Nile virus infection was described in a member of the laboratory staff where circumstantial evidence supported the hypothesis that infection was due to inhalation of infected material issuing from a defective Waring blendor. It was also shown that airborne infection could occur in animals under experimental conditions.

The purpose of this paper is to present some aspects of the pathogenesis of West Nile infection in mice exposed to a viral aerosol, studied through the use of titrations in animals, fluorescent-antibody technique and histological examinations.

MATERIALS AND METHODS

Virus.—The strain of West Nile virus and preparation of virus suspension for generating the aerosol have already been described (Nir, 1959).

Aerosol exposure and titration of tissues.—Batches of 30 Swiss albino mice (3 weeks old) in groups of 6 animals each, were exposed to an aerosol emanating from a Collison atomizer containing a West Nile virus suspension. Exposure to the virus lasted from 15 to 20 min. and the amount of virus inhaled by each animal was calculated, with the aid of Guyton's formula (Guyton, 1947), to be $2{\cdot}4 \times 10^4$–$1{\cdot}6 \times 10^5$ mouse intracerebral (mic) LD_{50}. At various intervals after exposure, 3 mice were killed by cervical dislocation, bled from the heart, and the livers, kidneys, spleens, cervical lymph nodes, lungs, olfactory bulbs and brains were removed. The nasal mucosae were either scraped off with a knife or collected with the nasal septa. Unless stated otherwise, samples were taken from each individual tissue for virus titrations as well as for fluorescent-antibody staining and histopathological examinations. The smaller samples were ground with glass powder in 0·5 ml. of physiological saline supplemented with 10 per cent heated rabbit serum (RSSP). The others were weighed and ground with diluent to make 10 per cent suspensions. Virus titrations were carried out by preparing tenfold serial dilutions of these suspensions in the diluent and inoculating 0·03 ml. intracerebrally into 4-week-old mice. Six mice were inoculated with each dilution. Animals were observed for 14 days and deaths which occurred during the first 3 days were not considered to be due to the virus. Virus titres expressed in mice LD_{50} per 0·03 ml. and based on the number

of dead and surviving mice after the observation period, were calculated in accordance with the method of Reed and Muench (1938).

Routes of inoculation.—In the preliminary experiments, groups of mice were exposed to the virus via the following routes : (1) Intranasal instillation, (2) Application on the cornea, (3) Ingestion per os, (4) Rubbing on to the skin around the snout, (5) Intratracheal inoculation. For the latter purpose, the mice were anaesthetized, a longitudinal slit was opened in the skin of the neck and the trachea exposed. The virus was inoculated directly into the trachea with the aid of a 0·25 ml. syringe fitted with a slightly bent 27-gauge needle. The skin was sutured and the animals returned to their cages.

Preparation of antiserum.—Swiss albino mice were inoculated intracerebrally with a West Nile virus suspension consisting of 20 per cent mouse brain in RSSP. On showing definite signs of illness, these mice were killed, the brains removed and ground in a mortar to make a 20 per cent suspension. Rabbits were then injected weekly by the intravenous route with one ml. of a 1/10 dilution of this preparation for a period of 6 weeks. A similar amount as a booster dose was again administered 6 weeks later. Ten days after the last injection, the rabbits were bled by cardiac puncture. Pooled sera tested by intraperitoneal neutralization test in young mice gave a neutralizing titre greater than six logarithmic units.

Labelling of antiserum.—The procedures followed have already been described (Winnick and Goldwasser, 1961). After removal of unbound stain by dialysis against buffered saline, the conjugate was adsorbed with acetone-precipitated mouse brain and liver powder. The final preparation was kept at −20° in 0·5 ml. portions until used.

Preparation of lissamine-rhodamine labelled bovine albumin.—Bovine albumin was labelled with lissamine-rhodamine for counterstaining purposes according to the methods described by other investigators (Chadwick, McEntegart and Nairn, 1958 ; Smith, Marshall and Eveland, 1959).

Preparation of tissue sections.—Tissue specimens of mice exposed to the virus aerosol were mounted on metal holders and dropped into an isopentane bath kept at −60°. Sections 5 μ. thick were cut in a cryostat refrigerated at − 20°. The sections were transferred onto slides, stuck onto the glass by rapid thawing, fixed in acetone for 5 min. at room temperature and dried in air. The slides were kept in a freezer at −20° until stained with fluorescent antibody.

Fluorescent staining.—The tissue sections were flooded with a mixture of fluorescein-labelled antiserum and lissamine-rhodamine stain diluted in 20 per cent normal mouse brain suspension. They were then placed inside a moist chamber in an incubator at 37° for 30 min. At the end of this period, they were washed first with 0·02 M-carbonate-bicarbonate solution and then in water. After drying in air, the sections were ready for examination in the fluorescence microscope. As a control for specificity of staining, the same fluorescent mixture mentioned above, diluted in 20 per cent West Nile virus infected mouse brain suspension, was used to stain identical tissue sections (Goldwasser and Kissling, 1958).

Histology.—Histopathological examinations were made on 5–7μ. thick sections obtained from frozen or formalin-preserved tissues. All the sections were stained with haematoxylin-eosin and the brain sections were also stained with Luxol fast blue.

EXPERIMENTAL

Preliminary experiments

The object of these experiments was to ascertain the most probable routes of infection when mice are subjected to a viral aerosol under the conditions described. Since these conditions make it necessary to consider the possibility of entry of the virus via the mouth, nostrils, eyes and the skin around the snout, the susceptibility of mice to infection by these routes was tested with large doses of virus. The results are shown in Table I.

Since mice did not respond even to the massive dose of 10^6 mic LD_{50} of virus when applied on the skin, eyes or by mouth, it was assumed that in the aerosol experiments where a high mortality occurs, viral invasion was either via the nasal mucosa or the lungs. Comparative virus titrations were performed on bloods of

TABLE I.—*Susceptibility of Mice to* 10^6 *mic* LD_{50} *of West Nile Virus Administered by Various Routes*

Route of Infection	Mortality*
Skin	0/10
Eyes	0/10
Mouth	0/10
Nose	11/12
Trachea	8/12

* Numerator = number died ; denominator = number inoculated.

mice inoculated with the same amounts of West Nile virus intranasally and intratracheally. Viraemia appeared within 24 hr. after intratracheal inoculation whereas no virus was detected in the blood before the 96-hr. titration after the intranasal inoculation of virus (Table II).

TABLE II.—*Virus Titres in Bloods of Mice Inoculated Intranasally and Intratracheally with* $1{\cdot}5 \times 10^6$ *mic* LD_{50} *of West Nile Virus*

Time after Inoculation (hr.)	Titre* of virus following	
	Intranasal inoculation	Intratracheal inoculation
4	0	0
24	0	1·3
48	0	1·9
72	0	0·8
96	1·2	1·3
120	0	0
144	2·5	0·9

* Expressed as the log of the reciprocals of the LD_{50} endpoint dilution. Means of titres of two separate samples from individual mice.

Aerosol experiments

The great majority of mice exposed to the aerosols showed specific signs of disease from the fourth day following exposure. The identity of the virus recovered from the brains of randomly selected mice was confirmed by neutralization tests with specific antiserum.

In those mice which were bled and killed at intervals after exposure, the following tissues were titrated : blood, liver, spleen, kidneys, adrenals, cervical lymph nodes, lungs, nasal mucosa and brain. The results are shown in Table III.

Immediately after exposure, demonstrable quantities were detected only in the lungs. Four hours after exposure, the amount of virus detectable was minimal. At the 24-hr. titration, while all the other tissues tested remained free from virus, the lungs showed a titre much higher than at the 4-hr. titration. It was estimated that at this time, the total amount of virus found in the lungs was somewhat greater than the amount of virus inhaled by the animals. This indicates that there was at least some local multiplication of virus. It was only at the 48-hr. titration that virus appeared in tissues other than the lungs. Samples of blood and spleen taken at this time contained small amounts of virus indicating that some virus had finally escaped into the general circulation, while none was detected as yet in the liver, kidneys, adrenals, cervical lymph nodes and nasal mucosa.

No antigen was detected with florescent-antibody techniques in most of the slides prepared from liver, spleen and kidney tissues taken at 0, 4, 24, 48, 72, 96, 120 and

TABLE III.—*Distribution of West Nile Virus in Selected Tissues of Mice at Various Intervals Following Exposure to the Virus in Aerosol Form*

Tissue	Time (hr.) 0	4	24	48	72	96	120	144
Blood	0*	0	0	0·2	0	0·6	0·5	0·2
Liver	0	0	0	0	0	0·2	0·3	1·1
Spleen	0	0	0	1·0	0·9	0·5	0·6	1·7
Kidneys	0	0	0	0	0	0	0·5	1·8
Adrenals	0	0	0	0	0	0	1·7	3·0
Cervical lymph nodes	0	0	0	0	0	0	0	0·3
Lung	1·0	0·6	2·6	4·0	4·5	4·1	4·2	4·1
Nasal mucosa	0	0	0	0	0·1	0·4	1·4	1·4
Brain	0	0	0	0·1	1·7	3·7	8·0	7·6

Titres of virus expressed as the log of the reciprocals of the LD_{50} endpoint dilution. Means of titrations from 4–6 mice.

* 0 = No virus detected in highest concentration tested.

144 hr. after exposure to the virus. Doubtful staining was obtained in occasional slides from spleen tissue taken at 144 hr. On the other hand, positive staining of antigen was noted, apparently in macrophages, in all the sections of lung tissue from 24 hr. after exposure to the virus aerosol. In the lung tissue sections taken at the 48 hr. period and later, many more cells seemed to contain antigen than in the 24-hr. specimens. The antigen appeared in the cytoplasm only (Fig. 1).

Small amounts of virus, detected by mouse inoculation, began to appear in the brain 48 hr. after exposure. Subsequently, multiplication of virus in the brain became evident and the titre increased steadily until it reached its maximum at 120 hr. after exposure. Antigen staining also appeared in some sections of the brain taken at the 48-hr. period. Antigen was seen in a few scattered cells only, here again localized in the cytoplasm (Fig. 2). In some of the neurones, viral antigen was observed also in the axons (Fig. 3).

It was of interest to learn which region of the brain exhibited the first signs of virus infection. Consequently, another group of 30 mice was exposed to an aerosol of West Nile virus. At given intervals after exposure, 3 mice were killed and the brains removed. The olfactory bulbs and portions of the centres of the pyriform areas of midbrain and cerebellum were cut from each brain. Part of each brain

EXPLANATION OF PLATES

FIG. 1.—Frozen mouse lung section stained with fluorescent antibody, 48 hr. after exposure to a West Nile virus aerosol. Antigen appears to be located in cytoplasm of macrophages. ×610.

FIG. 2.—Frozen section from an olfactory bulb of a mouse, 84 hr. after exposure to a West Nile virus aerosol. Stained with fluorescent antibody. Some cells contain antigen in the cytoplasm. ×610.

FIG. 3.—Frozen section of a mouse brain, 84 hr. after exposure to a West Nile virus aerosol. Stained with fluorescent antibody. At centre, a neurone with antigen in its axon. At bottom right, another cell containing antigen in its cytoplasm. ×610.

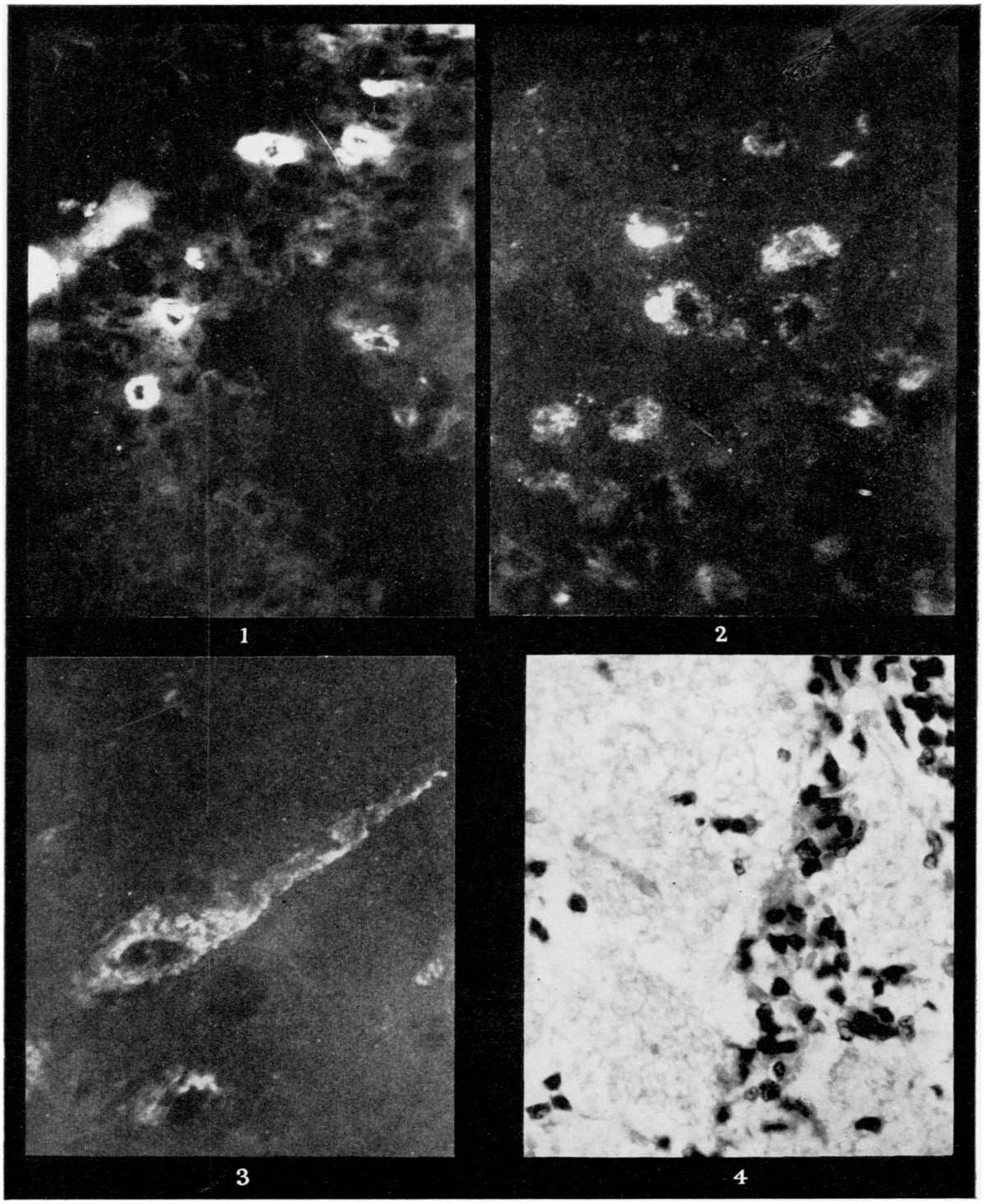
FIG. 4.—Group of inflammatory cells in olfactory bulb of mouse, between mitral-cell and glomerular layers, 84 hr. after exposure to the virus aerosol. H. and E. ×720.

FIG. 5.—Section of olfactory bulb showing degenerative changes in mitral cells, 120 hr. after exposure. H. and E. ×110.

FIG. 6.—Inflammatory infiltration under pia mater in section of hippocampus, 96 hr. after exposure. H. and E. ×265.

FIG. 7.—Section of capillaries in hippocampus with considerable perivascular infiltration, 96 hr. after exposure. H. and E. ×265.

FIG. 8.—Congested capillary and degenerated Purkinje cells in section of cerebellum, 144 hr. after exposure. H. and E. ×110.



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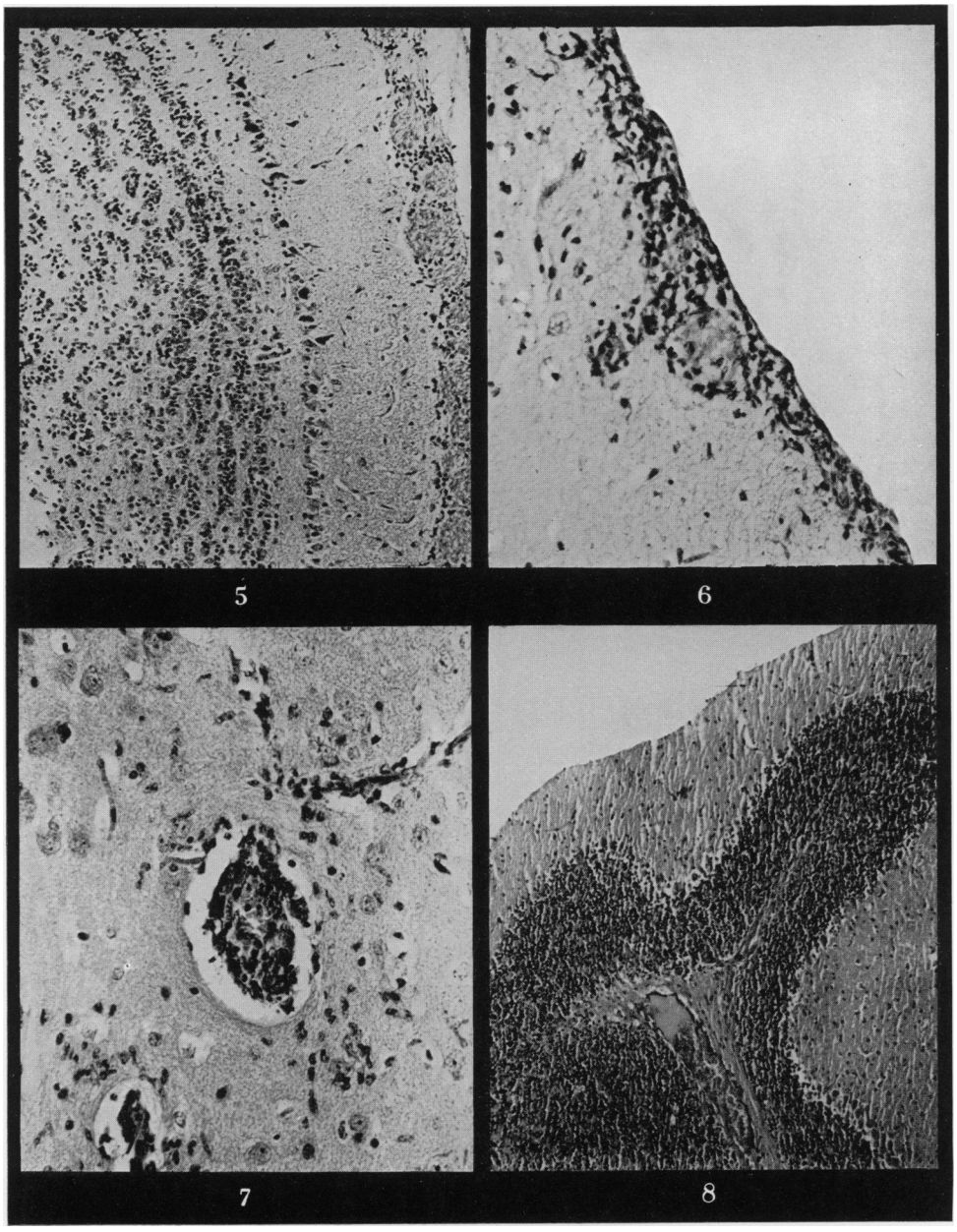

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tissue specimen was processed for histological examinations. The remainders were pooled according to their respective origins and titrated. The results which are summarized in Table IV indicate that virus first began to appear in the CNS in the olfactory bulbs.

TABLE IV.—*Titres of West Nile Virus in Olfactory Bulbs, Midbrain and Cerebellum of Mice exposed to the Virus in Aerosol Form*

Tissue	Time (hr.) 24	36	48	60	72
Olfactory bulbs	0	2·6	4·5	4·7	5·2
Midbrain	0	0	1·0	1·8	3·8
Cerebellum	0	0	<1*	0	2·4

Titres of virus expressed as the log of the reciprocals of the LD_{50} endpoint dilution obtained in pools of three samples.

* <1 = 1–2 mice succumbed out of 6 inoculated with the 10^{-1} dilution.

Antigen was first detected by the fluorescent-antibody technique in some of the sections from the olfactory bulbs taken 48 hr. after exposure. Subsequently all the sections from this tissue were found to contain antigen.

Histological findings

No significant changes were found in lung, liver, spleen and kidney tissues taken from 0 hr. up to 144 hr. after exposure to the virus.

Olfactory bulbs.—No significant pathological changes were seen in sections stained with hematoxylin-eosin and Luxol, from 0–72 hr. after exposure. After 84 hr. some inflammatory cells were seen between the mitral cell and glomerular layers of the bulb (Fig. 4). At 96 hr. there was round-cell infiltration in the pia mater, the olfactory bulb and the anterior pyriform area. Perivascular cuffing and some polymorphonuclear infiltration were also observed. Degenerative changes were also seen in the mitral cells of the olfactory bulb at 120 hr. (Fig. 5). Cellular degeneration and perivascular cuffing were marked at 144 hr.

Hippocampus.—No significant changes were seen in the hippocampus up to 84 hr. In the 96 hr. sections, an inflammatory reaction was seen in the pia mater which was thickened and infiltrated with inflammatory cells (Fig. 6). Perivascular cuffing (Fig. 7), degenerative changes and infiltration with lymphocytes and polymorphs were seen in scattered foci in the substance of the hippocampus.

Cerebellum.—Pathological changes in the cerebellum were first observed in the 144-hr. sections. The vessels were dilated and congested and there was some round-cell infiltration and degeneration of Purkinje cells (Fig. 8).

It is noteworthy that no pathological changes were seen in the nasal mucosa in any of the sections examined including those of 120 hr. after exposure.

DISCUSSION

Exposure of mice to an aerosol of West Nile virus resulted ultimately in a generalized infection of these animals, virus being found in varying amounts in all the tissues tested. Only the intranasal and the respiratory routes were found to be worthy of consideration after it was shown that mice were refractory to infection following administration of large doses of virus on the skin, the eyes or by mouth. Virus which was inhaled by the animals was found in the lungs immediately after

exposure and appeared consistently there in appreciable amounts thereafter. The fact that at the 24 hr. titration virus appeared in the lungs but in no other organs tested suggests that it found there its earliest site of multiplication. Other investigators who exposed mice to an aerosol of another arbovirus (tick-borne encephalitis virus) also found that following this route of infection, the virus multiplied first in the lungs where it persisted for the whole period of observation (Danes, Libich and Benda, 1962).

In the later stages, brain tissue gave the highest titres of infective virus. This is not surprising since pathological lesions in mice infected with West Nile virus are usually restricted to the central nervous system (CNS), regardless of the route of inoculation (Southam, Newman and Messore, 1961). Invasion of the CNS does not appear to stem from early multiplication of virus in the lungs. No virus was found in the cervical lymph nodes and no viraemia appeared in any of the exposed mice before virus appeared in the brain. This seems to rule out the possibility that virus appearing in the CNS was carried there from the lungs by blood or lymph circulation. Virus titrations of different parts of the brain seem to point to the invasion of the CNS along the olfactory pathway. Virus was found in the olfactory bulbs before it was detected in the brain proper. At the 36 hr. titration, in one experiment, virus at a significant level was demonstrated in olfactory bulbs of the exposed mice while none was found in midbrain and cerebellum of the same mice. Invasion of the CNS via the olfactory bulbs has been shown to occur with the closely related St. Louis encephalitis virus following nasal instillation in mice (Brodie, 1935).

It should be noted that no virus could be detected in the nasal mucosa of mice in the early hours of the infection. It seems that the virus particles adsorbed by the cells lining the nasal mucosa do not multiply there. This is in agreement with the findings of Peck and Sabin (1947) who, working with the St. Louis encephalitis virus, similarly observed that there was no evidence of virus multiplication in the nasal mucosa of mice which succumbed with encephalitis after nasal instillation of the virus.

In our hands, fluorescent-antibody technique proved to be less sensitive than mouse inoculation for the detection of virus. While the sections taken from tissues of all organs shown to be negative by the latter method were also negative by the fluorescent-antibody technique, some of the specimens which were shown to contain virus by mouse inoculation did not stain. It is worth mentioning in this connection the findings of Noyes (1955) who after making comparative titrations of the Egypt 101 strain of virus in human epidermoid carcinoma cells in cultures by means of fluorescent antiserum and intracerebral injection of mice concluded that the former was ten times more sensitive.

Histological examinations showed that although virus multiplication occurred in the lungs, it was not accompanied by any pathological changes. Lesions appeared mainly in the olfactory bulbs, the hippocampus and the cerebellum in that chronological order. The time of appearance of virus and of lesions did not coincide, lesions appearing 24–48 hr. after the virus could first be detected.

SUMMARY

The dissemination of virus in selected tissues of mice at different time intervals after exposure to an aerosol of West Nile virus was studied by the simultaneous use of animal titration, fluorescent-antibody and histological techniques.

No virus in significant amounts was found in the liver, spleen, kidneys, adrenals, cervical lymph nodes and nasal mucosa during the first 4 days following infection and no lesions were seen in these tissues at any time.

Proliferation of virus in the lungs was observed as soon as 24 hr. after infection. Fluorescent-antibody staining antigen was found in the cytoplasm of macrophages in this tissue. No pathological damage was seen on histological examination.

Maximal multiplication of virus took place in the CNS in which occurred the only lesions detected by histopathological techniques. First signs of the presence of virus appeared in the olfactory bulbs before its appearance in the midbrain and the cerebellum.

It is concluded that following exposure of mice to an aerosol of West Nile virus, invasion of the CNS probably occurs through the olfactory pathway.

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