Experimental encephalitis following peripheral inoculation of West Nile virus in mice of different ages

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SUMMARY

Experimental arbovirus infections of mice provide a convenient model to study factors which determine the occurrence or severity of encephalitis following extraneural infection with certain neurotropic viruses. Varying doses of West Nile or Powassan viruses were inoculated by intraperitoneal or intramuscular routes into mice of varying ages; individual variables were manipulated to influence the outcome of infection. Three patterns of pathogenesis were delineated: (1) Fatal encephalitis, preceded by early viraemia, and invasion of the central nervous system. (2) Inapparent infection, with no detectable viraemia and no evidence of central nervous system invasion. (3) Subclinical encephalitis, usually preceded by trace viraemia, with minimal transient levels of virus in the brain. In this latter type of subclinical infection with a potentially lethal virus, the immune response probably plays an important role in recovery.

INTRODUCTION

Human infections with neurotropic viruses are known to range in their manifestations from fatal encephalitis through aseptic meningitis to inapparent extraneural infection. It is well established that experimental arbovirus infections in mice also range from inapparent to lethal, following peripheral virus inoculation. Furthermore, the outcome of infection is influenced by a number of readily manipulated variables, including virulence and dose of virus, route of inoculation, and age of host (Albrecht, 1968; Cole & Wisseman, 1969; ElDadah, Nathanson & Sarsitis, 1967; Grossberg & Scherer, 1966; Johnson & Mims, 1968; Lennette & Koprowski, 1944). Thus experimental arbovirus infections can be used to delineate some of the factors which may determine the occurrence of encephalitis following peripheral virus infection.

The present investigation was designed as a series of pair comparisons, in each of which a single experimental variable was altered to convert inapparent into lethal infection. For each of these pairs, the comparative pathogenesis was studied, in an attempt to define factors critical to the outcome of infections.

MATERIALS AND METHODS

Animals

MBR/ICR albino Swiss outbred mice were obtained from a commercial breeder (Hazelton-Carbia, Laurel, Maryland). Pregnant mice were followed to determine exact age of young. The term 'newborn' is used throughout to refer to animals 1-3 days of age.

Virus

West Nile virus strain Egypt 101 (E101) had had approximately four egg followed by six newborn mouse brain passages (ElDadah *et al.* 1967). Powassan virus had had about eight newborn mouse brain passages (Thind & Price, 1969*b*). Viruses were inoculated intracerebrally (0.02 ml., without anaesthesia), intraperitoneally (0.05 ml., left lower quadrant), or intramuscularly (0.05 ml., gastrocnemius). All virus doses are expressed as newborn mouse intracerebral LD 50.

Titrations

For determination of 50% lethal (LD 50) and infectious (ID 50) end-points, eight animals were inoculated with each decimal dilution of virus. After 4 weeks observation, survivors were bled from the orbital plexus for serum antibody and challenged by intracerebral inoculation of 100 LD 50. Animals with antibody or resisting virus challenge were considered to have undergone inapparent infection (ElDadah *et al.* 1967).

Pathogenesis experiments

To follow the course of infection, four mice in each experimental group were killed daily. Heparinized bloods or sera were pooled for viraemia or antibody determinations, respectively. Following perfusion with saline, tissues from all animals were removed for immunofluorescent examination. For histological preparations 2-4 additional mice were killed at 2 and at 3 weeks after infection; in the case of lethal infections, animals were killed when moribund.

Heparinized bloods were tested for viraemia by intracerebral inoculation of newborn mice with decimal dilutions prepared in 0.75% bovine plasma albumin in phosphate-buffered saline. In those instances where no viral antigen was detected by immunofluorescence (Figs. 4B, 6B, 7B), tissues were also homogenized, centrifuged, and supernates inoculated into newborn mice (Cole & Wisseman, 1969; ElDadah & Nathanson, 1967).

Histological methods

For immunofluorescent (IF) staining, animals were perfused with sterile saline, organs removed, embedded in 10% gelatin, and 8 μ sections cut at -20°C . The direct fluorescent antibody method was used with Evans blue counterstain added (Cole, Nathanson & Rivet, 1970; ElDadah & Nathanson, 1967). Tissues routinely examined were: brain (parasagittal section), cross-section of thigh (muscle and peripheral nerve), liver, spleen and kidney. Immunofluorescence was graded sep-

arately for each tissue, according to a semiquantitative scale: 0 = none seen; 1 = a few scattered cells; 2 = 1 to 25 % cells stained; 3 = 25 to 75 % cells stained; 4 = over 75 % cells stained. Within each experimental group, observations were usually similar for different mice killed on the same day, and a median of four animals is recorded in the figures.

For light microscopy, mice were perfused with 10 % formalin containing 1 % acetic acid, paraffin sections were cut at 10 μ , and stained with haematoxylin and eosin (ElDadah & Nathanson, 1967).

Haemagglutination-inhibition (HI) antibody

Sera were treated with acetone and tested in microtitre plates with six units of antigen prepared by the sucrose acetone method (Clarke & Casals, 1958; ElDadah *et al.* 1967). Inhibition at a serum dilution of 1/20 or greater was considered to represent specific antibody (recorded as + in Figs. 2-7).

RESULTS

Age-specific titrations of West Nile and Powassan viruses

The upper half of Fig. 1 shows that West Nile virus had a high intracerebral titre in mice of all ages ($10^{8.3}$ LD₅₀/0.02 g. in newborn and 10^7 LD₅₀ in adult animals). Following intramuscular or intraperitoneal inoculation, the titre was high in newborns ($10^{8.3}$ LD₅₀/0.05 g.), but decreased with increasing host age. By the intramuscular route, no deaths occurred in mice age 4 weeks, and by the intraperitoneal route few deaths occurred in mice age 6 weeks or over.

Infectious titres (ID₅₀) were determined by testing survivors of titrations for haemagglutination-inhibition (HI) antibody, followed by intracerebral virus challenge with 100 LD₅₀. In newborn mice inoculated by any route, LD₅₀ and ID₅₀ were identical; that is, there was no evidence of sublethal infection. A few older mice surviving intracerebral inoculation had undergone sublethal infection, but the ID₅₀ was no more than $10^{0.5}$ greater than the LD₅₀. Following intraperitoneal or intramuscular injection, the age-specific ID₅₀ was about $10^{6.5}$ at 4 weeks of age and $10^{5.5}$ at 12 weeks of age. Thus, peripheral inoculation of West Nile virus regularly produced inapparent infections in older mice.

Powassan virus was included in the study, because it was known to possess high neuro-invasiveness following peripheral inoculation in older mice, in contrast to the low peripheral virulence of West Nile virus. The lower half of Fig. 1 shows that, following intraperitoneal or intramuscular inoculation, the LD₅₀ titre of Powassan virus in newborn mice was $10^{9.3}$, falling to 10^8 and to 10^6 in mice 4 and 12 weeks of age, respectively. By any route, the ID₅₀ of Powassan virus was only slightly greater than the LD₅₀, even in mice age 12 weeks.

Influence of experimental variables on pathogenesis

A systematic study of the course of infection was made for different inoculation routes in mice of varying ages, using both West Nile and Powassan viruses. The different combinations studied are listed in Table 1. Since this study was designed

to contrast pathogenesis under circumstances where a single experimental variable markedly influenced the outcome of infection, the list in Table 1 has been arranged to delineate the comparisons which were made. Figs. 2-7 follow the same scheme.

Fig. 1. West Nile and Powassan virus age-specific lethal (LD 50) and infectious (ID 50) titres in mice following intracerebral, intraperitoneal, and intramuscular inoculation. ID 50 based on HI serum antibody in survivors bled 4 weeks after infection, and on intracerebral challenge at same time with 100 newborn mouse i.c. LD 50 of homologous virus.

Effect of age

The pathogenesis of West Nile virus infection following intracerebral inoculation of 1000 LD 50 in mice age 1-3 days and age 4 weeks are presented in Fig. 2. At both ages a regularly fatal encephalitis occurred, and the data are shown for comparison with infection following peripheral inoculation, presented in Figs. 3-7.

Fig. 3 contrasts intraperitoneal inoculation of 1,000,000 LD 50 of West Nile virus in mice age 1-3 days and in mice 12 weeks of age. In newborn mice, virus appears in the blood by day 2 and in the brain by day 3, with most animals dying 5-7 days after inoculation. In animals age 12 weeks there was no mortality or signs of illness and, although viraemia was not detected, virus did invade the central nervous system. Viral antigen was detected in scattered individual neurones in brains examined 8 days after infection.

Table 1. *Summary of pathogenesis studies of experimental arbovirus infections in mice; pair comparisons of variables which influence the occurrence of lethal encephalitis**

Key variable in each pair comparison	Virus	Route of inoculation	Dose $\log_{10}$ LD 50	Age	Outcome	CNS invasion	Pathogenesis summarized in fig.
Age	West Nile	i.c.	3.0	1-3 days	Died	+	2
	West Nile	i.c.	3.0	4 weeks	Died	+	
Age	West Nile	i.p.	6.0	1-3 days	Died	+	3
	West Nile	i.p.	6.0	12 weeks	Survived	+	
Age	West Nile	i.m.	3.0	1 week	Died	+	4
	West Nile	i.m.	3.0	4 weeks	Survived	-	
Route	West Nile	i.p.	6.0	4 weeks	Died	+	5
	West Nile	i.m.	6.0	4 weeks	Survived	+	
Dose	West Nile	i.p.	6.0	4 weeks	Died	+	6
	West Nile	i.p.	3.0	4 weeks	Survived	-	
Virus	Powassan	i.m.	3.0	4 weeks	Died	+	7
	West Nile	i.m.	3.0	4 weeks	Survived	-	

* Virus dose in newborn mouse i.c. LD 50/inoculum.

Fig. 2. Course of West Nile virus infection in mice, following intracerebral inoculation of 1000 newborn mouse i.c. LD 50. Comparison of lethal infections in animals age 1-3 days and age 4 weeks. See methods for immunofluorescence (IF), grading scale. Viraemia: $\log_{10}$ newborn mouse i.c. LD 50/0.02 ml. whole blood. Haemagglutinating-inhibiting antibody (HIAB): +, titre of 1/20 or greater.

Fig. 3. Course of West Nile virus infection in mice, following intraperitoneal inoculation of 1,000,000 newborn mouse i.c. LD 50. Comparison of lethal infections in animals age 1-3 days and inapparent infection in animals age 12 weeks. See caption for Fig. 2.

Fig. 4. Course of West Nile virus infection in mice, following intramuscular inoculation of 1000 newborn mouse i.c. LD 50. Comparison of lethal infection in animals age 1 week and inapparent infection in animals age 4 weeks. Peripheral tissues of animals age 4 weeks were also negative on subinoculation. See caption for Fig. 2.

Fig. 4 presents another age comparison. Death regularly occurred 5–8 days after inoculation in 1-week-old mice injected intramuscularly with 1000 LD₅₀ of West Nile virus. Viraemia was detected on days 2–5, and immunofluorescent staining revealed widespread infection in brain and other tissues, beginning about 3 days after infection. By contrast, mice age 4 weeks had inapparent infections, following intramuscular inoculation of 100 LD₅₀. Furthermore, direct titrations and immunofluorescent observations failed to reveal virus either in blood, or in a variety of extraneural tissues including the inoculated muscle.

Fig. 5. Course of West Nile virus infection in mice age 4 weeks, following inoculation of 1,000,000 newborn mouse i.c. LD₅₀. Comparison of lethal intraperitoneal infection and inapparent infection following intramuscular inoculation. See caption for Fig. 2.

Route of inoculation

Fig. 1 indicates that mice 4 weeks of age were not killed by intramuscular injection of any dose of West Nile virus, but did succumb to intraperitoneal injection of large virus doses. Fig. 5 shows a comparison of fatal intraperitoneal and sub-clinical intramuscular inoculation of 1,000,000 LD₅₀. The critical difference appears to be the occurrence of viraemia, which was irregularly detected on days 2–7 following intraperitoneal injection, with invasion of brain about day 5, followed by fatal encephalitis. By contrast, intramuscular injection was followed by minimal viraemia detectable only on day 4; invasion of the central nervous system occurred late (minimal amounts of viral antigen seen on days 8–10), and only an occasional mouse (3 of 42) developed clinically apparent encephalitis.

Virus dose

A comparison of lethal and sublethal infection, under conditions where the critical variable was virus dose, is shown in Fig. 6. Mice age 4 weeks, injected by the intraperitoneal route, all died after a dose of 1,000,000 LD₅₀, while those given 1000 LD₅₀ survived. The larger dose produced viraemia, with subsequent invasion of the brain; the smaller dose failed to produce detectable viraemia or evidence of

Fig. 6. Course of West Nile virus infection following intraperitoneal inoculation in mice age 4 weeks. Comparison of lethal infection produced by large inoculum (1,000,000 newborn mouse i.c. LD 50) and inapparent infection produced by smaller inoculum (1000 LD 50). For animals given 1000 LD 50 peripheral tissues (including peritoneal lymph nodes) were tested by subinoculation since they were negative for immunofluorescent viral antigen. +, titre ≥ 10 LD 50/0.02 g. See caption for Fig. 2.

Fig. 7. Course of West Nile and Powassan virus infections in mice age 4 weeks, following intramuscular inoculation of 1000 newborn mouse i.c. LD 50. Comparison of lethal Powassan virus infection and inapparent West Nile virus infection. Peripheral tissues of animals given West Nile virus were also negative on subinoculation. See caption for Fig. 2.

central nervous system invasion, but virus was found (by subinoculation) in spleen and peritoneal lymph nodes on days 4–6.

Viral virulence

To delineate the influence of viral virulence, West Nile was compared with Powassan virus, as shown in Fig. 7. Mice 4 weeks of age were inoculated intramuscularly with 1000 LD₅₀ of each virus. Under these conditions West Nile virus produced neither detectable viraemia nor infection of the central nervous system, and virus was not detected in a variety of tissues. By contrast, Powassan virus produced a marked viraemia, present from days 2–8, with invasion of brain, spinal cord, and other tissues, first detected on day 2 or 3, followed by death of all animals on days 5–7.

Histological evidence of encephalitis

Each of the groups of animals in Figs. 2–7 were examined for evidence of encephalitis. Lethal infections (Figs. 2, 3A, 4A, 5A, 6A, 7A) were accompanied by readily apparent histological changes, including leptomeningitis, perivascular cuffs, and focal infiltrates; neuronal necrosis or outfall were patchy and most frequent in cerebral cortex and hippocampus. In instances where there was no evidence of viral invasion of the CNS (Figs. 4B, 6B, 7B), there was no histological evidence of encephalitis. In circumstances where a subclinical CNS infection occurred (Figs. 3B, 5B) a mild meningo-encephalitis was seen in some brains, with little detectable neuronal outfall; failure to see changes in some animals may have been due to transient nature of inflammation (animals were killed at 14 and 21 days after infection), to the limited number of sections examined, or to absence of neuro-invasion.

DISCUSSION

This study distinguishes three categories of infection following peripheral arbovirus inoculation: (1) widespread infection with subsequent overwhelming involvement of the brain; (2) minimal immunizing infection without involvement of the CNS; and (3) an intermediate category where virus reaches the CNS, but undergoes only limited replication. These markedly different courses of infection pose two obvious questions which require further consideration: first, what are the differences in the extraneural phases of infection which determine whether CNS invasion occurs; and second, why, in some instances, does a potentially lethal neurotropic virus regularly invade the CNS and yet give rise to only a limited subclinical infection?

Extraneural phase of infection

Recent reviews by Johnson & Mims (1968) and by Albrecht (1968) describe the sequence of events in arbovirus encephalitis. There is early multiplication in extraneural tissues, followed by viraemia, with subsequent spread into the brain. Albrecht (1968) summarized a number of studies suggesting that variations in the extraneural phase of infection could be an important determinant in the occurrence of CNS invasion. The present study is consistent with this view. Thus, *age* comparisons (Fig. 3, 4) show a much greater viraemia in young than old mice, which

correlates with the distribution of immunofluorescence in tissue and indicates that extraneural virus replication was much more widespread and intense in young animals (Fig. 4). Other variables being constant, *route* of inoculation can determine outcome; Fig. 5 indicates that intraperitoneal injection produces an earlier and more prolonged viraemia than intramuscular injection, perhaps reflecting differences in the relative efficiency with which the virus inoculum reaches susceptible cells (cf. Figs. 4B, 6B). Under conditions where there is a delicate balance between parasite and host, virus *dose* can be critical. Fig. 6 shows that a large dose can initiate viraemia; a small dose fails to produce detectable viraemia (minimal levels of virus were detected in peritoneal lymph nodes and spleen), and there is no subsequent CNS invasion.

The high peripheral virulence of Powassan virus in older mice (in contrast to West Nile virus) correlates with much higher viraemia titres (Fig. 7). This may solely reflect the greater ability of Powassan virus to replicate in those extraneural sites which seed virus into the circulation. However, arboviruses can also vary markedly in the rates with which they are cleared from the circulation, which in turn could influence viraemia potential (Postic, Schleupner, Armstrong & Ho, 1969).

Virus replication in the central nervous system

Of the three types of infection seen in this study, two appear relatively readily understood. Lethal encephalitis is characterized by cytolytic infection of a high proportion of neurones and glial cells; death may occur so rapidly that histological changes as seen by conventional light microscopy are not fully developed (Albrecht, 1968; ElDadah & Nathanson, 1967). At the other end of the range, infections in which virus does not become established in the brain necessarily produce no changes in the CNS.

Of most interest is the intermediate category of infection in which virus reaches the CNS, but undergoes an abortive subclinical cycle of replication and then disappears. Under appropriate experimental conditions, this pattern occurs with regularity (Figs. 3B, 5B). Since West Nile virus injected intracerebrally, even in small inocula, is potentially capable of producing lethal encephalitis in mice of any age (Fig. 1), the occurrence of sublethal encephalitis requires explanation.

It is clear from this and prior studies (Albrecht, 1968; ElDadah & Nathanson, 1967) that in an abortive CNS infection a small number of widely scattered neurones (and possibly glia) are infected; these cells are destroyed, usually with concomitant production of mild inflammatory changes. Thus, infection is clearly established throughout the CNS, but fails to progress even though a potentially susceptible substrate is available.

The occurrence of subclinical CNS infection following extraneural inoculation with highly virulent arboviruses is probably a relatively common laboratory phenomenon, since findings similar to ours have been reported for a number of arboviruses: louping-ill (Doherty, 1969), Langat (Thind & Price, 1969*a*; Webb *et al.* 1968), Japanese encephalitis (Huang & Wong, 1963) and Venezuelan equine encephalitis (Gleiser, Gochenour, Berge & Tigertt, 1961). These descriptive studies of pathogenesis indicate that virus invasion of the CNS occurs later during abortive

infection than it does during lethal infection. In the present study, virus was first detected in the CNS on day 8 in abortive infections (Figs. 3B, 5B) and on days 3–5 (Figs. 3A, 4A, 5A, 6A, 7A) in lethal infections. This suggests that, if CNS invasion is sufficiently delayed, host defence mechanisms can outrace the virus and abort the infectious process (Mims, 1964).

One of the defence mechanisms which plays an important role in abortive encephalitis is the immune response. The use of immunosuppressive techniques to convert abortive arbovirus infection into lethal encephalitis (Cole & Nathanson, 1968; Nathanson & Cole, 1970; Weiner, Cole & Nathanson, 1969) provides ample evidence for this view.

Since a number of group B arboviruses are both efficient inducers of interferon as well as sensitive to its action (Finter, 1967), the interferon response could also be of importance in the outcome of infection. However, several relevant studies of arbovirus infections in mice indicate that interferon production appears to be directly related to virus replication, more interferon being detected in tissues of animals in which virus titres are highest (Cole & Nathanson, 1968; Cole & Wisseman, 1969; Subrahmanyam & Mims, 1966; Vainio, Gwatkin & Koprowski, 1961). In our view, such findings fail to suggest a significant role for interferon in this experimental model. A more important role has been proposed for interferon in studies of genetically determined differences in the susceptibility of mice to West Nile virus (Baron, 1970; Hanson, Koprowski, Baron & Buckler, 1969).

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