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Tick-borne encephalitis

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Abstract

Tick-borne encephalitis (TBE) is one of the most dangerous human infections occurring in Europe and many parts of Asia. The etiological agent *Tick-borne encephalitis virus* (TBEV), is a member of the virus genus *Flavivirus*, of the family *Flaviviridae*. TBEV is believed to cause at least 11,000 human cases of encephalitis in Russia and about 3000 cases in the rest of Europe annually. Related viruses within the same group, *Louping ill virus* (LIV), *Langat virus* (LGTV) and *Powassan virus* (POWV), also cause human encephalitis but rarely on an epidemic scale. Three other viruses within the same group, *Omsk hemorrhagic fever virus* (OHFV), *Kyasanur Forest disease virus* (KFDV) and Alkhurma virus (ALKV), are closely related to the TBEV complex viruses and tend to cause fatal hemorrhagic fevers rather than encephalitis. This review describes the clinical manifestations associated with TBEV infections, the main molecular–biological properties of these viruses, and the different factors that define the incidence and severity of disease. The role of ticks and their local hosts in the emergence of new virus variants with different pathogenic characteristics is also discussed. This review also contains a brief history of vaccination against TBE including trials with live attenuated vaccine and modern tendencies in developing of vaccine virus strains.

Keywords: *Tick-borne encephalitis virus*; *Omsk hemorrhagic fever virus*; Chronic tick-borne encephalitis; Biphasic milk fever; Pathogenesis; Live attenuated vaccine; Bioterrorism

1. Classification of *Tick-borne encephalitis virus* (TBEV)

Tick-borne encephalitis (TBE) is one of the most dangerous neuroinfections in Europe and Asia. It is caused by TBEV, previously known as Russian Spring and Summer Encephalitis (RSSE) virus and was discovered in 1937 during an expedition in Far-East Russia led by Lev Zilber searching for the etiological agent of acute encephalitis associated with tick bites.

Under the most recent taxonomic scheme (Heinz et al., 2000), TBEV belongs to the tick-borne flavivirus group, family *Flaviviridae*, genus *Flavivirus*. There are two other groups of viruses within the *Flavivirus* genus, the mosquito-borne viruses and viruses for which there are no known vectors (NKV). The tick-borne flaviviruses are sub-divided into a Mammalian group and a Seabird group, and TBEV is classified as one of the species within the Mammalian group. The TBEV species includes three sub-types, namely Far Eastern (previously RSSE), Siberian (previously west-Siberian) and Western European (previously Central European Encephalitis, CEE) virus. In this chapter we will follow the latest classification scheme although in referred papers, published before the new scheme the old names for virus subtypes have been used. Virus species are indicated in italics whereas subtype viruses are not italicised.

Many viruses antigenically related to TBEV were isolated across Europe, Asia and Canada and collectively they became known as the TBEV serocomplex (Porterfield, 1975; Calisher, 1988), now defined as the Mammalian group of tick-borne flaviviruses. Besides TBEV, the Mammalian group includes *Louping ill virus* (LIV), *Langat virus* (LGTV), *Powassan virus* (POWV), *Omsk hemorrhagic fever virus* (OHFV), *Kyasanur Forest disease virus* (KFDV), *Kadam virus* (KADV), *Royal Farm virus* (RFV) and its subtype Karshi virus and *Gadgets Gully virus* (GGYV). In addition, Alkhurma virus (ALKV) was recently recognised and is sufficiently closely related to KFDV to be considered a subtype of this virus (Charrel et al., 2001). *Louping ill virus* is genetically the most closely related to TBEV, and also shares the same tick vector but its distribution on the sheep-rearing hillsides of Scotland, Wales, England and

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Ireland distinguishes it from TBEV which is found in forests across Europe and Asia. In general, the frequency of fatal encephalitic disease following infection with any other of these Mammalian tick-borne virus species is less than that seen following infection with TBEV strains.

Phylogenetic analysis of these viruses has essentially proven the validity of the previous serological classification schemes (Porterfield, 1975; Calisher, 1988) and enabled predictions that the tick-borne viruses probably diverged from the mosquito-borne flaviviruses not more than 5000 years ago (Zanotto et al., 1995, 1996a; Gould et al., 2001).

2. Molecular–biological characteristics

Mature virions are about 50 nm in diameter and are composed of an electron dense core surrounded by a lipid bilayer containing two envelope glycoproteins, E (envelope) and M (membrane). Intracellular (immature) virions contains a precursor prM protein and the cleavage of prM to M occurs during the exit of virions from cells (Murphy, 1980). The core consists of a single stranded RNA genome of positive polarity (~11 kb in length) and a capsid protein (C). The E protein is the major surface protein of the viral particle. It interacts with cell receptors and mediates virus-cell membrane fusion. In mammalian hosts it also induces virus-neutralising antibodies that play an important role in the establishment of protective immune responses (Heinz, 1986).

The genomic RNA contains one open reading frame (ORF) and encodes a polyprotein of about 3400 amino acids that is cleaved into three structural proteins (C, M and E) and seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) by cellular and viral proteases. During the infectious cycle, the NS3 (helicase) and NS5 (RNA-dependent RNA polymerase) proteins form polymerase complexes that are probably associated with membranes through the nonstructural protein NS1 and NS2A (Lindenbach and Rice, 2001). The NS1 protein is associated with membranes as hexameric ring-like particles about 10 nm in diameter (Gritsun et al., 1988, 1989, 1990; Flamand et al., 1999) that under denaturing conditions form stable dimeric molecules (Winkler et al., 1988). The NS1 protein which was previously called “soluble antigen”, induces protective immune responses against flaviviruses (Gould et al., 1986; Schlesinger et al., 1986; Cane and Gould, 1988; Jacobs et al., 1992). The NS3 protein in association with the NS2B protein provides virus-specific serine protease activity for the cleavage of newly synthesised virus polyprotein. The non-structural proteins NS4A and NS4B probably provide appropriate orientation of the polyprotein within intracellular membranes, thereby ensuring correct cleavage and functioning of polymerase complexes (Lindenbach and Rice, 2001).

The open reading frame of all flaviviruses is flanked by 5′ (about 130 nucleotides) and 3′ untranslated regions (UTR) (400–700 nucleotides). RNA in these regions forms secondary stem-loop structures that probably serve as *cis*-acting elements for genome amplification, translation, or packaging (Gritsun et al., 1997; Proutski et al., 1997b; Rauscher et al., 1997).

3. Virus stability

Because of the lipid envelope, TBEV is readily inactivated by organic solvents and detergents. Nonionic detergents, such as Triton X, solubilise the entire envelope, releasing M and E proteins; whereas sodium deoxycholate appears to remove only E, leaving M associated with the nucleocapsid. The envelope protects the genome from cellular nucleases, and naked nucleocapsids, released by detergent treatment, are degraded by ribonuclease (Russell et al., 1980). Nevertheless, purified naked RNA is infectious following direct intracerebral injection of mice, although care needs to be taken to avoid its degradation (Gritsun and Gould, 1995).

Flavivirus infectivity and viral hemaglutinin are optimally stable at pH 8.4–8.8 (Karabatsos, 1980). Nevertheless, TBEV has been reported to preserve at least residual infectivity over the broader pH range 1.42–9.19 being roughly comparable with the stability of enteroviruses (Pogodina, 1958). Although at acidic pH the E protein of TBEV virus undergoes specific conformational changes that reduce virus infectivity (Heinz et al., 1994), virions still remain infectious in curdled milk and gastric juice (Gresikova-Kohutova, 1959; Pogodina, 1958) and this explains why TBEV may infect via the alimentary route.

Flaviviruses are rapidly inactivated at 50 °C, 50% of infectivity being lost in 10 min. Total inactivation of virus suspended in blood or other protein solutions occurs within 30 min at 56 °C. On the other hand, ultra low temperatures preserve infectivity almost indefinitely (Gould, 1995). Aerosols, either as water droplets or in the form of powder, present an infectious hazard in the laboratory and should be avoided by appropriate containment. Flaviviruses are stable for at least 6 h in liquid aerosol suspension at room temperature and 23–80% humidity (Karabatsos, 1980). In freeze-dried form they survive almost indefinitely at room temperature. Flaviviruses are inactivated by ultraviolet light, gamma-irradiation, and disinfectants, including 3–8% formaldehyde, 2% glutaraldehyde, 2–3% hydrogen peroxide, 500–5000-ppm available chlorine, alcohol, 1% iodine, and phenol iodophors. The tick-borne viruses appear to be relatively more resistant to these forms of treatment than mosquito-borne viruses (Burke and Monath, 2001).

4. Routes of TBEV infection

Under natural conditions humans walking through the dense vegetation in forests are most likely to become infected with TBEV following the bite of an infected tick. Ticks quest for a bloodmeal between spring and autumn

usually on warm sunny days. They climb onto vegetation and wait for a passing animal (or human). The carbon dioxide given off by the animal attracts ticks. Serological surveys suggest that between 70 and 95% of human infections in endemic regions are sub-clinical (asymptomatic) (Shapoval, 1976, 1977; Pogodina et al., 1986).

The incidence of clinically expressed forms of TBE varies annually and in different regions and is thought to depend on several factors, such as number of humans encountering infected ticks, the length of time spent by the tick feeding on the host, the density of ticks, the infectivity of ticks and the concentration of virus within individual ticks (Alekseev and Chunikhin, 1990; Korenberg and Kovalevskii, 1995, 1999; Leonova, 1997; Korenberg et al., 2001). Nevertheless, the most important factors contributing to the incidence of disease is the abundance of ticks containing a high dose of infectious TBEV that occur in so-called nuclei of natural foci where tick concentration is much higher than in the surrounding areas (reviewed by Korenberg and Kovalevskii, 1999). It was calculated that in an endemic region, about 1–2% of the local human population who came into contact with infected ticks over the epidemic season developed disease (Korenberg and Kovalevskii, 1995; Leonova, 1997).

Another natural route of human TBEV infection is associated with the consumption of goat milk which is drunk in many parts of the old Soviet Union as a substitute for cow milk. It has been experimentally demonstrated that TBEV can be isolated from the milk of goats for 5–25 days following infection and the infectivity survives in various milk products such as yoghurt, cheese and butter (Shapoval, 1977). Drinking infected milk may cause biphasic milk fever. TBEV virions are stable for up to two hours in normal gastric juice at pH 1.49–1.80 and in gastric juice with reduced acidity (pH 1.87–2.21) (Pogodina, 1958). In gastric juice taken from humans after a meal (pH 2–7) the virus infectivity is stable for 2 h. Milk foods move out of the stomach quite quickly (the first milk consumed reaches the duodenum within minutes and after 1.5–2 h there is no milk in the stomach). Hydrochloric acid is secreted in the stomach between 45 and 60 min after consumption of the milk. It was therefore concluded that the human digestive tract is an efficient route of infection and this was confirmed in experiments with mice which became infected after they were fed orally with TBEV (Pogodina, 1960). There is also independent evidence of oral infection of red grouse chicks by LIV (E.A. Gould, unpublished data). When fed LIV-infected ticks, grouse chicks developed fatal encephalitic disease. Moreover, *Japanese encephalitis virus*, a mosquito-borne flavivirus, has been shown to infect mice when fed orally (Ramakrishna et al., 1999).

In addition to conventional transmission by infected ticks, OHFV which is closely related to TBEV, is also believed to infect humans through direct contact with infected muskrats which are hunted and processed for fur by local farmers (Kharitonova and Leonov, 1985) (vide infra). The belief is that the virus infects by penetration through cuts on the skin of the people processing the animals. Alternatively, it is known that TBEV complex viruses are secreted into the faeces and urine of infected mice (Pogodina, 1960) and therefore this might also be a transmission route for OHFV.

Cases of laboratory TBEV infections have also been associated with accidental needle-stick injuries during injections of animals with virus. Aerosol infections among laboratory personnel were reported in laboratories when glass bottles containing high virus concentrations were accidentally broken in walk-in incubators (Scherer et al., 1980). In these latter cases encephalitis developed but there were no neurological sequelae. Finally, TBEV complex viruses are quite stable when preserved as freeze-dried samples (Gould, 1995) and since it is known that they could infect via aerosols, glass ampoules containing freeze-dried virus are a potential source of infection if they are broken in an exposed environment. Despite these comments, it is worth noting that there is no published evidence of such problems arising with any category three or four viruses that have been freeze-dried in glass ampoules.

5. Ecological and epidemiological characteristics of TBEV infections

In the natural environment, TBEV is maintained in a cycle involving ticks and wild vertebrate hosts. The natural habitat is the forests of Europe and Asia where ticks can find high humidity in the dense undergrowth of the forests. Ticks remain infected throughout their life cycle and transmit virus to uninfected ticks when co-feeding on small wild rodents. Although other larger animals, including birds, deer and horses also serve as hosts for ticks, they are not known to be important hosts for virus transmission between ticks. The viruses have adapted to the physiological and behavioral characteristics of ticks, particularly with regard to blood-feeding, blood-meal digestion and moulting (Nuttall et al., 1994). For many years, it was assumed that persistence of tick-borne viruses in ticks occurred through viremic, transstadial or transovarial transmission. However, it is now known that there is another important mechanism for supporting virus circulation. Transmission of virus from infected to non-infected ticks occurs when they co-feed on the same host and it is not necessary for the host to develop a detectable viremia (Labuda et al., 1993). The local skin site where the tick feeds is now known to be an important focus of viral replication where migratory cells provide a vehicle for virus transmission from infected to uninfected co-feeding ticks. These data support the view that viremia is probably a product, rather than a prerequisite, of tick-borne virus transmission (Labuda et al., 1996).

The epidemiology of TBE is closely associated with the ecology and biology of ticks, the areas of their dissemination and periods of feeding activity. Ticks inhabit specific foci in forests with enhanced moist vegetation where local small and large wild animals supply a blood meal for them.

The climatic conditions (temperature, moisture, vegetation) that occur only within specific geographical zones (limited from north and south) provide a discontinuous distribution of ticks and create at least 20,000–30,000 natural foci of TBE infections across the Northern Hemisphere from Europe to Japan (Korenberg and Kovalevskii, 1999).

The maximum incidence of human infections coincides with seasonal peaks of feeding activity of the ticks. Peak feeding times for the dominant tick species in Central Europe, *Ixodes ricinus*, occur between May and June and also between September and October and these are the periods of peak incidence of human disease. *Ixodes persulcatus* which are mainly distributed in the Ural, Siberia and the Far East have one peak feeding period between May and June. This coincides with the maximum incidence of TBE human infections in these regions.

The incidence of TBE varies from year to year in different geographic regions (Korenberg and Kovalevskii, 1999). The Pre-Ural and Ural region as well as Siberia have the highest records of hospitalised cases (Zlobin and Gorin, 1996; Korenberg and Kovalevskii, 1999). During the 1950s and 1960s, TBE was recorded primarily in forest workers, reaching 700–1200 cases annually. The epidemiological situation deteriorated following "Perestroika" (Zlobin and Gorin, 1996; Korenberg and Kovalevskii, 1999), with up to 11,000 recorded cases per year amongst urban dwellers who become infected when they visit the local forests, on holiday, fishing, collecting flowers, berries, mushrooms or even working in their gardens. As the medical infrastructure changed, following "Perestroika", fewer people received immunisation against TBE and the use of pesticides also ceased. It is also of note that currently, 35–45% of all infected persons are children from 2.5 to 12 years old. Finally, there is also accumulating evidence that a high proportion of the rural population has either no or very low antibody levels against TBEV despite the fact that they have a very high likelihood of being exposed to the virus. This observation has not yet been adequately explained, but it has been suggested that since the same problem seems to exist with other virus diseases such as measles and poliomyelitis, exposure to agrochemicals might have reduced the immunocompetence of the population.

TBE also occurs in many parts of Western and Central Europe and Scandinavia, particularly, Austria, Croatia, Czech Republic, Estonia, Finland, Germany, Hungary, Latvia, Lithuania, Poland, Slovak Republic, Slovenia, Sweden, and Switzerland, with many recorded cases annually (Blaskovic et al., 1967; Roggendorf et al., 1981; Gresikova and Calisher, 1988; Lindgren and Gustafson, 2001; Ormaasen et al., 2001). The total number of annual cases in Western European countries has averaged 3000 for the last 5 years. Detailed statistics can be obtained from the following website http://www4.tbe-info.com/epidemiology.

Recent studies are beginning to throw new light on the geographic distribution of TBEV. Phylogenetic analysis of the tick-borne flaviviruses showed that the genetic distance between each of the recognised TBEV and closely related viruses correlated closely with their geographic distance from a point in Scotland, UK where the most recently diverged TBEV complex viruses were found (Zanotto et al., 1995, 1996b; Gould et al., 2001). This clinal evolution and the geographic distribution of these viruses have been re-analysed using global satellite data and another correlation has been described. It has now been suggested that the regions in Europe and Southern Scandinavia where TBE exists may correlate with climatic conditions that favor co-incident co-feeding transmission between *Ixodes ricinus* larvae and nymphs (Randolph et al., 1999). On the basis of these ideas it should be possible in the future to predict with reasonable certainty the geographic regions in which TBEV should be capable of producing serious epidemiological problems amongst either animals or humans.

6. Clinical manifestations of TBE

Regardless of disease severity, the incubation period of TBE on average lasts between 7 and 14 days. In some patients, early symptoms include fatigue lasting 1–2 days, with pain in the neck, shoulders, and the lower back. Headache may also be observed at this early stage. The classical symptoms appear suddenly. Patients can often recall the exact hour of onset. There is a sudden elevation of temperature 38–39 °C, a feeling of nausea, accompanied by vomiting, once or twice per day. The muscular pains become severe and are localised in the neck, shoulder, lower spine and limbs. Sometimes fasciculations and a sense of numbness occur in one of the limbs. In some patients, meningeal symptoms such as neck stiffness, develop during this period. There may also be shortness of breath, flushing of the face, neck and upper part of the body.

Russain medical authorities recognise that TBEV produces a variety of clinical symptoms which can be differentiated into the following forms of TBE (141, 1990).

(i) *Febrile form*. This usually occurs with complete recovery of the patient. There are no neurological symptoms and there is no obvious damage to the CNS. This form occurs in about one third of all recognised TBEV infections. The febrile period, during which the temperature may reach 39 °C, can last from a few hours to 5 days.
(ii) *Meningeal form*. This is the most common form of TBE with a similar onset to the febrile form but with more severe symptoms. Patients complain of very strong headaches and a feeling of nausea. There is frequent vomiting, and the patient becomes photophobic suffering pain in the eyes. The fever lasts for 7–14 days with gradual recovery.
(iii) *Meningoencephalitic form*. This form occurs less frequently but is more severe and associated with damage to the CNS. The patients are very weak, drowsy, hallucinate frequently and sometimes become unconscious.

The symptoms include fibrillar contractions, bradycardia, bradykinesia, stomach bleeding, hyperkinesia, hemiparesis and hemiplegia. Some patients subsequently experience epileptic fits. Up to 30% of cases are fatal and in survivors, particularly in older people, hemiplegia is irreversible. Convalescence is very slow, with signs of nervous exhaustion, malaise and frequent mood changes.

(iv) *Poliomyelitic form*. This is characterised by a prodromal period when patients experience fatigue, periodic muscle contractions, a sense of weakness or even numbness in one of the limbs, that later develops as a paralytic disorder. Between the first and fourth day of the first febrile period or during the first to third day of the second febrile period paresis of the neck, shoulder and upper limbs intensifies, sometimes lasting up to 2 weeks, and occasionally for several months. Typically, there is wrist drop, and in individuals asked to stand, their head hangs down or droops (referred to in Russia as the syndrome of a "hanged head"). By the end of the second or third week the muscles begin to atrophy. Paresis or paralysis of the lower limbs is quite rare. The course of the disease is always severe, the recovery is very slow, and only about one half of the patients show partial recovery from the neurological injuries. Slow progressive deterioration of the patient is common following this form of the disease.

(v) *Polyradiculoneuritic form*. This form is characterised by damage to, and pain in, the peripheral nerves. The course of the disease is biphasic, the first phase commencing within 3–7 days with fever, headache, elevated temperature (38–39 °C), insomnia, vomiting, muscular pains in the limbs. There is then a period of 7–14 days when the temperature returns to normal. The second phase starts with an elevated temperature and symptoms of damage to the CNS, meningeal and focal neurological symptoms are observed. Recovery is normally complete.

(vi) *Chronic form*. This form of TBE has been described in patients from Siberia and Far East (Russia) and has never been reported in Europe. It is believed to be associated with the Siberian subtype of TBEV (Votiakov et al., 1978; Pogodina et al., 1986). There are two types of chronic TBE. The first is often defined as the long-term sequelae of any of the acute forms of TBE, i.e. when symptoms that developed during the main course of the disease deteriorate for months or even years. Although evidence of virus presence in these cases is difficult to produce, post-mortem examination in many cases revealed recent perivascular infiltration in the brain and spinal cord, that pathologists interpret as the consequence of virus persistence in the infected organ. In rare cases the presence of virus after many decades of acute TBE had been confirmed by molecular hybridisation (T.V. Frolova, personal communication). However, chronic TBE can begin without the typical acute disease symptoms. In many of these cases, the development of neurological symptoms, following the bite of the infectious tick, may take years. There are a variety of clinical pictures relating to chronic TBE infections in humans, with variations in the incubation period, the time of onset of neurological symptoms, and the patient survival time after the onset of disease. Clinical symptoms include Kozshevnikov's epilepsy, progressive neuritis of the shoulder plexus, lateral sclerosis, dispersed sclerosis, a Parkinson-like disease and progressive muscle atrophy (Shapoval, 1976; Votiakov et al., 1978). Frequently the physical deterioration is accompanied by mental deterioration leading to severe dementia and/or death. The etiology of chronic forms of TBE in both seropositive and seronegative patients has often been confirmed by virus isolation following the onset of symptoms (Levina and Pogodina, 1988; Pogodina et al., 1986; Shapoval, 1976).

Doctors also distinguish *hyperkinesia* and *epileptoid syndrome*, that are associated with the meningeal, meningoencephalitic, poliomyelitic and polyradiculoneuritic forms of TBE and also with biphasic TBE. Hyperkinesia is encountered quite frequently (one-fourth of patients), especially among patients under 16 and is characterised by spontaneous regular contractions (myoclonus) in individual groups of muscles in paretic limbs. It may arise during the acute period of TBE or persist as Kozhevnikovs epilepsy. Epileptoid syndrome is frequently observed in patients who have a record of epilepsy.

The incidence of these different forms of TBE varies in different regions; for example, in Siberia about 80% of TBE infections that result in illness present as a fever without neurological sequelae, although hospitalisation and special medical care are frequently required. Paralytic forms are seen in about 7–8% of cases and Kozshevnikov' epilepsy (a form of chronic TBE), in about 4–5% (Zlobin and Gorin, 1996). Approximately 7% of patients die following acute encephalitis but the proportion of fatalities varies in different regions and the possible reasons for this will be considered below. Although the incidence of TBE in Far Eastern Russia is lower than in Siberia, fatality and disability rates are higher, in some regions reaching 60% (Pogodina et al., 1986). In countries such as Austria the fatality rate was 1% when vaccination was not readily available and fatal infections are now rarely recorded in European countries where immunisation has been actively encouraged (Kunz et al., 1976; Barrett et al., 1999).

7. Association of pathogenesis with TBEV subtype

Despite their wide geographic distribution, different strains of TBEV are antigenically very closely related and for much of the early research period, it was believed that only one virus circulated across Europe, Siberia and the Far

East. Nevertheless, the existence of two different pathogenic variants, CEE and RSSE eventually became recognised (Chumakov et al., 1944) and the differences between these viruses were subsequently confirmed in serological tests (Clarke, 1960). A third subtype of TBE virus that was previously called west-Siberian was then proposed, based on clinical signs in humans and geographical location. Antigenic analysis confirmed the differentiation of this virus from the CEE and RSSE subtypes (Pogodina et al., 1981a). Nucleotide sequencing has now validated the distinctness of three viruses despite their close antigenic and biological similarity (Mandl et al., 1989; Pletnev et al., 1990; Safronov et al., 1991; Gritsun et al., 1993a, 1997; Wallner et al., 1995, 1996; Ecker et al., 1999) and defined them as three subtypes of the same TBEV species, namely European, Siberian and Far Eastern (Heinz et al., 2000).

Although direct evidence has not been produced, the opinion based on statistical estimation is that human infections with TBEV of different subtypes result in the development of clinical manifestations of varying severity (Votiakov et al., 1978). Human infection with a Far Eastern subtype viruses results in the most severe form of CNS disorder with a tendency for the patient to develop focal meningoencephalitis or polyencephalitis accompanied by loss of consciousness and prolonged feelings of fatigue during recovery. In the most severe forms there is major damage to neurones in different parts of the brain and spinal cord. Case fatality rates of 20–60% have been recorded (Pogodina et al., 1986). During these epidemics, the slow progressive form of TBE is rarely observed and the disease in children is more severe than in adults (reviewed by Votiakov et al., 1978).

In contrast, TBE viruses isolated in the west-Siberian region of Russia (Siberian subtype) characteristically induce a less severe acute period and a high prevalence of the non-paralytic febrile form of encephalitis. Case fatality rates rarely exceed 6–8%. Instead, there is a tendency for patients to develop chronic TBE as will be defined in more detail below.

Encephalitis produced by European subtype viruses is biphasic with fever during the first phase and neurological disorders of differing severity, during the second phase, which occur in 20–30% of patients. In contrast with severe Far Eastern subtype virus infections, those following infection by European strains are usually milder, mostly without sequelae; case fatality rates are often as low as 1–2% and the disease in children is less severe than in adults (Burke and Monath, 2001).

There are many experimental data that demonstrate pathogenic differences between Far Eastern and European viruses on the one hand and Far Eastern and Siberian TBE strains on the other (reviewed by Votiakov et al., 1978).

Comparative analyses between Far Eastern and European viruses, producing different forms of experimental TBE have been carried out on monkeys and sheep (Votiakov et al., 1975, 1978). Whereas Far Eastern virus directly infected and damaged neurons in the brain, resulting in severe encephalitis, European virus initially did not replicate in or damage neuronal cells even after intracerebral inoculation of virus. Instead, the primary target of European virus was lymphoid tissue and the virus subsequently appeared in the brains, 6–9 days after inoculation (in cerebellum predominantly) of those animals that developed encephalitis. On the basis of this research it was concluded that Far Eastern viruses frequently produce severe encephalitis with fatal outcome because they have a tropism for neurons of the brain and spinal cord and the rapid degeneration of the neurons occurs due to direct replication of the virus in these cells. In contrast European viruses do not produce severe infection in the neurons. Damage to the neurons occurs only in some animals as a secondary inflammatory effect arising from infection of glial cells (Votiakov et al., 1978).

Prior to nucleotide sequencing, two biological markers, i.e. different pathogenic characteristics and small differences in serological cross-reactivity were most frequently used to differentiate European and Far Eastern viruses. Additionally, and perhaps quite unfairly ignored, these two virus subtypes have different sensitivities to dextran sulfate, implying that European and Far Eastern viruses could have different receptor specificities which could explain their different pathogenicities (Dzhivanian and Lashkevich, 1970; Dzhivanian et al., 1974). It is also worth considering the fact that the differences between European and Far Eastern viruses can be explained by their association with different species of ticks i.e. *Ixodes ricinus* and *Ixodes persulcatus*, respectively (vide infra).

Separate experiments using monkeys and Syrian hamsters demonstrated pathogenic differences between Far Eastern and Siberian subtypes of TBEV (Pogodina et al., 1986). The prototype virus of Siberian group, Aina, was differentiated serologically from both European and Far Eastern viruses (Pogodina et al., 1981a). A significant etiological role for Aina serotype viruses in both acute and chronic forms of TBE was supported by serological analysis of patients analysed months or years after the onset of the disease. Another well-characterised virus strain in this serogroup is Vasilchenko (Vs; Frolova et al., 1982a; Gritsun and Gould, 1998). The general features of all these strains specified as Siberian subtypes was their ability to produce slowly developing pathology and asymptomatic infections (reviewed in (Pogodina et al., 1986; Shapoval, 1976)). Initially experimental evidence of the possible association of chronic forms of TBE with Siberian TBEV strains came from modelling chronic infection in monkeys (Pogodina et al., 1981b–d; Fokina et al., 1982; Frolova and Pogodina, 1984a). For these forms of disease the characteristics were a prolonged incubation period, progressive development of neurological symptoms followed by paralysis of the limbs or partial recovery of movement functions. Asymptomatic infections produced by these viruses have also been demonstrated. During this type of infection TBEV invaded the same organs of the host as was reported for acute encephalitis (Fokina et al., 1982). In the early stages of infection (3–19 days) virus was

isolated mainly from the CNS. After 45 days it was not possible to isolate virus from the CNS and later it could be identified only in the internal organs.

The ability of Siberian viruses to persist and produce chronic disease has been also demonstrated in Syrian hamsters. The strain of Siberian TBEV, Vs, was largely used to model chronic TBE in monkeys and Syrian hamsters. This virus was isolated in the Novosibirsk region in 1961 by Kvetkova from the blood of patient with a non-paralytic form of TBE (Pogodina et al., 1986). In monkeys, Vs produced a broad variety of clinical diseases including chronic forms that developed either after non-lethal encephalitis or after asymptomatic infection. The descendant of Vs virus, called Vs-383, was isolated on Day 383 post-infection from an experimentally infected monkey that developed non-lethal encephalitis with stable paralysis of one of the front limbs (Malenko et al., 1982). It was subsequently demonstrated that Vs-383 had become more virulent for Syrian hamsters (51% lethality compared with 5% for Vs virus).

Two groups of animals were inoculated either with Vs or Vs-383 and monitored for 2 years (Frolova et al., 1982a,b; Frolova and Pogodina, 1984b). The descendant persistent viruses were isolated during this period from healthy animals and from animals that developed chronic encephalitis a long time after the experimental infection. In some animals the isolation of virus became possible only after stress-simulating hormones such as adrenaline or immunosuppressants, such as cyclophosphamide, prednisolone or vincristine had been administered. Each activated the replication of latent TBE virus, causing sickness in the previously healthy hamsters about 2 years after the original virus inoculation, directly producing evidence that TBE virus persists in apparently healthy animals.

8. Tick-borne encephalitis associated with LIV, LGTV and POWV

Besides TBEV, three other tick-borne flavivirus species within the Mammalian tick-borne group, LIV, LGTV and POWV also cause encephalitis in humans and/or animals but they do not produce significant epidemic outbreaks and, therefore, compared with TBEV have attracted less attention.

Louping ill virus (LIV) is the only flavivirus that is known to be present in the United Kingdom. This virus is transmitted to vertebrate hosts by *Ixodes ricinus* and causes a fatal encephalomyelitis in sheep and red grouse (*Lagopus lagopus scoticus*) (Timoney, 1972; Reid and Boyce, 1974). A similar disease has also been observed in sheep and goats in other European and Scandinavian countries but because the viruses in each country can be distinguished from LIV by nucleotide sequencing, each one has been assigned its own designation according to the country in which it was first recognised, hence the names Spanish sheep encephalomyelitis (SSE), Greek goat encephalomyelitis (GGE) and Turkish sheep encephalomyelitis (TSE; Gao et al., 1993; Marin et al., 1995). In common with its closest genetic relative TBEV, LIV may also be transmitted between infected and non-infected ticks which co-feed on vertebrate hosts without necessarily developing a viremia (Jones et al., 1997). However, viremic transmission undoubtedly occurs when ticks feed on non-immune (i.e. susceptible) sheep.

There have been very few reported cases of encephalitis due to LIV in humans, mostly among laboratory personnel (Davidson et al., 1991). The clinical picture for humans infected with LIV is very similar to that produced by European subtypes of TBEV. The first phase of disease is characterised by fever, lasting 2–11 days, followed by remission lasting 5–6 days and then the reappearance of fever and meningoencephalitis lasting 4–10 days, usually with full recovery. Although the virus is potentially a serious threat to humans on the British mainland, exposure in the natural environment is rare because the disease only occurs on the sheep-grazing uplands in Scotland, northern England, North Wales and Devon where the ticks are able to survive and reproduce in the thick, moist undergrowth. Sheep and deer provide an excellent source of blood for the ticks. Surprisingly, there is little evidence of LIV in the forests where other tick-borne pathogens such as the agent of lyme disease *Borrelia burgdorferi*, are known to prosper.

Langat virus (LGTV) was isolated in Malaysia and neighbouring Thailand from pools of ticks of *Ixodes granulatus* and *Haemaphysalis* spp. (Smith, 1956). It is not known to cause overt disease in rodents in their natural environment, but young laboratory mice inoculated intracerebrally develop encephalitis. Although there are no registered cases of human disease associated with this virus, specific antibodies against LGTV have been detected in the sera of local people. The virus is completely non-virulent for adult mice following subcutaneous or intraperitoneal inoculation and for primates following intracerebral inoculation. In the few cases of encephalitis that arose following vaccination with live attenuated LGTV-based vaccines (see below), the encephalitis was similar to that seen following infection with typical European strains of TBEV.

Powassan virus (POWV) circulates in South Dakota, the Western United States, Western Canada and Siberia (Lvov et al., 1974; Isachkova et al., 1978; Leonova et al., 1980; Johnson, 1987). The virus causes a similar severe encephalitis in humans and experimental primates as that produced by TBEV (Frolova et al., 1981, 1985), but some differences between the lesions in the CNS caused by POWV and TBEV were described (Leonova et al., 1991). In Canada and the USA, POWV causes severe encephalitis in humans with a high incidence of neurological sequelae and up to 60% case fatality rate. In Far Eastern Russia POWV infections were described as milder than those produced by TBEV. Other research provided evidence of latent infection with POWV in humans, domestic pets (cats, dogs, goats) and local rodents (Woodall and Roz, 1977; Mahdy et al., 1979; Leonova et al., 1987b).

In Far Eastern Russia, POWV co-circulates with TBEV and mixed infections with both viruses have been shown to occur (Leonova et al., 1987b). Among patients with encephalitis, antibodies to TBEV were detected in 69%, to POWV in 4% and to both viruses in 4% of cases. Antibodies to POWV and to both TBEV and POWV also had been found in healthy people bitten by ticks and patients with the chronic progressive form of TBE (Leonova et al., 1980).

In Canada, POWV had been isolated from *Ixodes cookei* that feeds mainly on groundhogs (*Marmota monax*), *Ixodes angustus* which often bite humans and cats and *Ixodes scapularis* and *Dermacentor variabilis* which frequently bite humans and dogs (Mahdy et al., 1979). In Russia, POWV has been isolated from other tick species, namely *I. persulcatus*, *H. neumanni*, *H. consinna* and *D. silvarum* (Lvov et al., 1974; Krugliak and Leonova, 1989) and replication in different tick species is believed to be a selection factor for different POWV strains (vide infra). Local rodents also probably play an important role in selection of different POWV variants and in laboratory experiments POWV has been shown to replicate in wild rodents *A. peninsulae Thomas*, *C. rufocanus Sund*, *A. agrarius Pall* and *M. fortis Butchen* (Leonova et al., 1987a). Interestingly, the virus has been isolated from mosquitoes (Kislenko et al., 1982) and in contrast to the other tick-borne virus species, POWV has an extra amino acid in the envelope E protein sequence, D_{336}, that is shared by mosquito-borne flaviviruses (Mandl et al., 1993; Gritsun et al., 1995). Phylogenetic analysis shows that POWV diverged as the most ancestral lineage of the Mammalian tick-borne viruses (Zanotto et al., 1995).

9. Role of tick and rodent species in selection of TBEV variants

Ixodes ricinus is the dominant hard tick species across Europe and the most epidemiologically important vector for European TBEV and LIV. Far Eastern and Siberian subtypes of TBEV are transmitted predominantly by *Ixodes persulcatus* that comprises 80–97% of all tick species in the Urals, Siberia and the Far East region of Russia. In some regions of Europe these two species overlap. However, other tick species, i.e. *Dermacentor pictus*, *Dermacentor silvarum* and *Hyalomma concinna* have also been associated with local TBE outbreaks in some areas of Siberia and the Far East, where *I. persulcatus* is not the predominant species (Zlobin and Gorin, 1996). *Tick-borne encephalitis virus* has also been isolated sporadically from at least 15 other ticks species and also from many other parasites (fly, flea, lice, mosquitoes) (Smorodintsev and Dubov, 1986; Zlobin and Gorin, 1996), but they are not believed to play a significant role in virus transmission to humans.

Evidence that the incidence of TBE correlates with the level of *I. persulcatus* and *H. concinna* in natural foci of Far East Russia was presented by (Leonova, 1997). There was also a correlation between disease severity and the relative prevalence of *I. persulcatus* or *H. concinna*. A high prevalence of *I. persulcatus*, correlated more frequently with severe and fatal cases wherea milder cases were recorded in years when *H. concinna* was the dominant tick species. When these tick species were at equivalent levels in any one focus, there was a relatively similar proportion of severe and mild forms of TBE and it was therefore proposed that these ticks select high and low-virulence TBEV strains (Leonova, 1997). The grey-sided vole *Clethrionomys rufocanus* appeared to be a host for both high- and low-virulence strains, whereas the common vole *Microtus arvalis* was only associated with low-virulence strains, implying that rodents also provide selective pressures on TBEV evolution.

The influence of ticks on the biological properties of TBEV has also been demonstrated in laboratory experiments (Khozinskaya et al., 1985; Dzhivanian et al., 1988, 1991; Labuda et al., 1994). Limited numbers of consecutive passages of TBEV or POWV solely in ticks resulted in the selection of virus variants that differ from the parent virus in biological characteristics. Following subsequent re-adaptation to mice the virus recovered its original phenotype, suggesting that phenotypic changes, resulting from selection, had affected the viral envelope protein. Indeed sequencing of the envelope E glycoprotein showed one amino acid difference between the original and tick-adapted viruses (Labuda et al., 1994).

The possible association of disease pathogenicity (hemorrhagic fever or encephalitis) with vector species (*Aedes* spp. or *Culex* spp., respectively) has been noted for mosquito-transmitted flaviviruses, suggesting that selection by the arthropod could influence the pathogenic properties of the viruses for humans (Gaunt et al., 2001). It is therefore possible to speculate that different pathogenic properties of different TBEV subtypes or other tick-borne virus species (LIV, POWV, LGTV) might be the result of adaptive evolution of viruses to different tick species. Nevertheless, the mammalian host might also influence short-term replication of the virus in the local skin site during co-feeding of infected and non-infected ticks (Labuda et al., 1996). Co-feeding transmission can also occur on immune hosts (Jones et al., 1997; Labuda et al., 1997) and this could provide the opportunity for selection of natural virus escape mutants that indeed are known to be present among tick-transmitted flavivirus populations (Gao et al., 1994). These observations could have important epidemiological implications relating both to the survival of TBE virus in animals and to the pathogenicity of the viruses for humans.

A mechanism of selection of viruses with different pathogenic characteristics was suggested on the basis of a systematic comparison of flavivirus E protein sequences (Gritsun et al., 1995). It was demonstrated that the distribution of amino acid variations in the virus receptor envelope E protein does not occur randomly but is concentrated as 19 distinct hypervariable short amino acid clusters to which different biological properties could be assigned,

i.e. antigenic differences, point mutations between vaccine and wild-type strains, altered tropisms and neutralisation escape mutants. It was concluded that the clusters represent surface-orientated amino acid stretches, so-called "hot spots" for selection where casual mutations do not destroy the critical elements of the E protein structure. The combination of amino acids in these clusters might determine the "pathogenic face" of the particular virus isolates and can be considered as genetic markers. The surface-orientated position of the clusters on the virion surface was subsequently confirmed by crystallographic analysis of the E protein (Rey et al., 1995). The adaptation of TBEV to specific mammalian cells was also accompanied by multiple amino acid substitutions that mapped to different clusters (Mandl et al., 2001). Whilst not excluding the possibility that adaptation of TBEV to ticks might go along with mutations in other genes, it is possible that selection of tick-adapted variants is associated with mutagenesis in the E protein clusters. This could define how flaviviruses with different pathogenic characteristics, emerge.

10. Biphasic milk fever

Biphasic milk fever, as a form of TBE which results from oral infection was first identified in the European part of Russia between 1947 and 1951. Typical TBE infections that are associated with tick bites, are acquired randomly, whilst during milk fever epidemics whole families contract TBE (Shapoval, 1989). It was eventually realised that this form of disease was associated with goat milk containing infectious TBEV. Goats were shown to develop sub-clinical TBE infections following tick bites and to become the source of TBE in the group of people consuming their milk (Popov and Ivanova, 1968; Korenberg and Pchelkina, 1975). Later reports also demonstrated TBEV transmission to humans through non-pasteurised milk of cows and sheep (Gresikova et al., 1975; Leonov et al., 1976).

The course of milk fever is biphasic, similar to TBE produced by European strains of TBEV although some differences were observed. The milk fever starts suddenly, with rapid elevation of temperature up to 38–39 °C, severe headaches and pains in the waist, neck and calf muscles and leg joints. The other symptoms include nausea, vomiting, insomnia, fatigue. The high temperature persists for 5–10 days and then gradually drops. The condition of the patient improves, but 6–10 days later a second phase occurs with elevated temperature that continues for up to 10 days. All symptoms listed above also occur in the second phase of the disease but with more intensity. Paresis or paralysis are never observed with this form of TBE, nevertheless meningeal symptoms are found for a small proportion of patients although there is full recovery. In summary there are three main differences between TBE associated with goat milk (milk fever) and tick bite, as follows (Smorodintsev, 1954; Burke and Monath, 2001).

1. The biphasic form is dominant in milk fever, whereas in tick-associated TBE, the biphasic form represents about 20–30% of all TBE infections.
2. The non-severe meningoencephalitis with slightly developed meningeal and parenchymal symptoms is limited only to milk fever, whereas the conventional clinical manifestations of tick-associated TBE are aseptic meningitis, meningoencephalitis, meningoencephalomyelitis, or meningoencephaloradiculitis.
3. Following milk fever, patients have a very high probability of recovering (almost 100%) without neurological sequelae, whereas tick-associated encephalitis results in some disability and death.

Taking into consideration the similar geographical location (European part of Russia and Western Europe) of viruses isolated from patients with milk fever or classical TBE, it can be concluded that both forms of infection are produced by the same virus strains and biphasic milk fever is regarded as an epidemiologically variant form of TBE rather than a disease etiologically associated with a particular strain of TBEV. The differences in clinical manifestation between TBE contracted by tick bite or via the alimentary tract are most likely explained by differences in the immunological response that depend on the route of virus penetration and the initial concentration of virus.

The biphasic milk fevers that are associated with milk have also been observed in Siberia (Leonov et al., 1976) and Far Eastern Russia (Popov and Ivanova, 1968; Vereta et al., 1991) regions where *I. persulcatus* is the dominant tick species.

Biphasic TBE, not associated with consumption of milk, is encountered in different parts of Russia with variable frequency: in west-northern areas of Europe—75%, in northern Europe—35–55%, in Siberia—19–23%, in Far East Russia—6–14% (Shapoval, 1955). In Far East Russia the second phase of disease is much milder in comparison with the biphasic meningoencephalitic form in Europe. Biphasic illness is considered to be a relapsing form of TBE and represents the clinical expression of continuous virus activity. It was demonstrated that in different patients, high titer antibodies develop at various times after the onset of the disease and therefore re-appearance of TBE might result from failure of the host, with a reduced immune response, to inactivate virus completely. In some cases, these relapses occur many times in the patient and this is considered to be a strong implication for chronic TBE.

11. Tick-borne flaviviruses causing hemorrhagic fevers

There are three recognised tick-borne flaviviruses (species within the Mammalian tick-borne virus group) that cause hemorrhagic fever in humans, *Omsk hemorrhagic fever virus* (OHFV), *Kyasanur Forest disease virus* (KFDV) and

recently identified Alkhurma virus (ALKV; Charrel et al., 2001) that will probably be designated a subtype of KFDV in the future.

11.1. Omsk hemorrhagic fever virus (OHFV)

The first descriptions of Omsk hemorrhagic fever were presented in 1941–1944 but, as a unique disease associated with virus closely related to TBEV, it was recognised in 1947 (reviewed by Lvov, 1988; Belov et al., 1995). Subsequent sequence analysis has confirmed the close phylogenetic relationships of OHFV with other tick-borne flaviviruses (Gritsun et al., 1993b). Epidemic foci characteristically occur in the Omsk and Novosibirsk regions of West Siberia, associated with the distinctive landscape of these regions, i.e. the forest-steppe and nearby lakes that are inhabited by muskrats. Muskrats are important sources for epidemic outbreaks in humans. Detailed epidemiological and ecological research has led to the conclusion that the muskrat probably selects and amplifies the most virulent strains of OHFV. Conventionally, transmission of virus to humans during outbreaks occurs through the bite of an infected tick. However, in the case of OHFV, it is believed that infection often occurs during direct exposure to the virus when the trapped muskrats are skinned. Epidemics are also believed to occur amongst the muskrats which are susceptible to OHFV probably because they were introduced into Siberia from Canada to replace extinct stocks. However, it has never been demonstrated that the previous Russian muskrats were resistant to OHFV. It is therefore possible that the extinction of muskrats could have been partly due to OHFV.

The distinctive feature of muskrat-associated outbreaks is the absence of the asymptomatic forms of disease that were observed quite often in individuals infected following the bite of a tick. This is probably because the virulence of the virus increased during passage through the muskrats.

The clinical symptoms of OHF in humans are different from other TBE infections. The onset is marked by fever, headache, myalgia and cough, which progress to bradycardia, dehydration, hypotension, and gastrointestinal symptoms. The most marked pathological signs of the disease are focal visceral hemorrhages in the mucus of the nose, gums, uterus and lungs. The clinical symptoms also include diffuse encephalitis which disappears during the recovery period. Convalescence is usually uneventful without residual effects; fatal cases have been registered, but rarely (0.5–3%) (Kharitonova and Leonov, 1985). During the period 1946–2000 at least 1344 cases of OHF were diagnosed (Busygin, 2000). Nevertheless, serological surveys suggest that more regular exposure of humans to virus occurs and that OHFV also produces a relatively mild form of disease, i.e. fever without hemorrhage and also asymptomatic infections, except under those circumstances when the muskrat supports virus circulation (Busygin, 2000).

The apparent emergence and decline of this disease provides us with an example of how human activity can perturb the evolutionary relationships between different vertebrate and invertebrate species in natural communities. Epidemics of OHF occurred between 1946 and 1947 and then abruptly ceased during the following years. This has been explained by the situation that arose in Russia after the end of World War II. Muskrats were re-introduced into the Omsk region in 1928 from Canada to replace the extinct species, but the outbreaks of OHF in humans handling these infected animals were observed in the 1940s. Before this time, OHFV had circulated in the natural environment among a variety of indigenous tick species and their hosts, including *Dermacentor reticulatus* that bites humans and the narrow-sculled vole (*Microtus gregalis Pall*) that inhabits open uncultivated areas. After World War II, there was a shortage of manpower in Russia and large areas of arable land were left unfarmed, providing ideal conditions for *D. reticulatus* and the narrow-sculled vole to increase to very high levels. Muskrats also increased in density during this period and became the source of the human virulent variant of OHFV. Subsequently, as the land began to be farmed again, there was a reduction of ticks in these areas and therefore the incidence of disease also decreased. It is now believed that the exclusive role of *D. reticulatus* and the narrow-sculled vole in association with muskrats was only an episode in the epidemiology of OHF. However, outbreaks in muskrats still occurred and serological surveys established that contacts of humans with OHFV also continued. Therefore, the virus continued to circulate although it is now primarily found in areas surrounding lakes as opposed to the farmland-associated disease in the 1940's. It was concluded that as long as the farmland continues to be cultivated, it is unlikely that there will be major tick-associated human epidemics in the future, although there will still be outbreaks associated with the fur-trade, i.e. through muskrats as a non-vectored transmission. Recent data support this prognosis. It was observed that during epidemic outbreaks of OHF during 1988–1997 in the Novosibirsk and Omsk regions, involving 165 cases, 10 of them were associated with ticks and 155 with muskrat hunters and poachers (reviewed by Busygin, 2000).

OHFV has also been isolated from mosquitoes, particularly *Aedes flavescens* and *Aedes subdiversus* that were trapped in regions where OHF outbreaks were registered among local hunters and muskrats during 1988–1991. Infected mosquitoes were only found in local regions surrounding the lakes where epidemics were occurring amongst the muskrats. It is worth noting that strains of OHFV isolated from ticks or mosquitoes differed in their neuroinvasiveness for laboratory mice, with lower indexes being recorded for mosquito-isolated strains. It has therefore been concluded that they are likely to be less important vectors of OHF (Busygin, 2000).

11.2. Kyasanur Forest disease virus (KFDV)

Kyasanur Forest disease virus was first isolated in 1957 in the Shimoga District of Karnataka State in India.

Serologically, the virus was shown to be a member of the tick-borne encephalitis complex and this was subsequently confirmed by sequencing (Venugopal et al., 1994). The appearance of hemorrhagic fevers among humans was connected to a large number of deaths with hemorrhagic symptoms, amongst monkeys. It was linked with the impact of deforestation (for increased cattle grazing) that may have prolonged the time that indigenous monkeys spend on the forest floor, leading to increased exposure to KFDV-infected ticks in the undergrowth. The most abundant tick species in this region is *Haemaphysalis spinigera* but the virus has also been isolated from seven other different species of *Haemaphysalis*, *Dermacentor* and *Ixodes* ticks. In humans the virus produces severe hemorrhagic fevers with a 2–10% fatal outcome (Work and Trapido, 1957). There have been anecdotal reports that KFDV is carried long distances by migratory birds. However, neither epidemiological nor serological evidence of this has been forthcoming. Nevertheless, the idea that related hemorrhagic fever viruses might exist outside India has now been substantiated (see below).

Alkhurma virus has been isolated several times since 1995, from the blood of patients with severe hemorrhagic fever in Saudi Arabia. Among 16 patients, 4 had lethal outcome. Sequence analysis revealed the close genetic relationship of this virus to KFDV (Charrel et al., 2001) suggesting that a group of closely related Mammalian tick-borne viruses circulates in India and the neighboring countries, at least as far west as Saudi Arabia and even perhaps, in terms of evolutionary origins, into Africa. It is also worth commenting that two other genetically closely related flaviviruses, *Karshi virus* (KSIV) and *Royal Farm virus* (RFV) are known to circulate in Uzbekistan and Afghanistan (Gould et al., 2001). They have not been reported to cause hemorrhagic fever in humans but this could be partly related to the regions in which they arise. Their close genetic relatedness to KFDV and ALKV should not be ignored.

11.3. Reasons for different clinical manifestations

Genomic sequencing of OHFV, KFDV and ALKV (Charrel et al., 2001; Gritsun et al., 1993b, 1995; Venugopal et al., 1994) demonstrated that they are quite closely related to neurotropic tick-transmitted flaviviruses but this comparison did not reveal any specific peptides or amino acids that could be implicated in virus targeting different tissues. We have already discussed the possibility of emerging viruses with different pathogenic characteristics due to the evolution pressure from different tick/rodent species and this speculation can also be applied to the mechanisms of emerging viruses that produce hemorrhagic disease instead of encephalitis. Indeed, OHFV and KFDV have been isolated from tick species different from those that transmit neurotropic viruses (vide supra) and therefore it is possible that the appearance of specific pathogenic characteristics was the result of adaptation of these viruses to particular tick species and/or their hosts. Genetic variants containing epitopes in the hypervariable clusters of the E protein (Gritsun et al., 1995) or in the non-structural protein NS1 that mimic blood or vascular proteins, might be selected. Such variants might trigger autoimmune reactions in the vertebrate host leading to the development of hemorrhagic disease (Falconar, 1997).

12. Control of TBE: prospects for live attenuated vaccine

12.1. Inactivated vaccines

Attempts at immunoprophylaxis against TBEV were first made in Russia in the early 1940s using inactivated vaccine prepared from TBE-infected mouse brains. Since then several generations of inactivated vaccine were produced and tested in human clinical trials (Smorodintsev and Dubov, 1986; Zlobin and Gorin, 1996; Leonova, 1997). The protective properties of inactivated vaccine are associated mostly with virions, particularly with the envelope protein. The development of inactivated vaccines was focused on improved immunogenicity and reduced allergic reactions. This was achieved by concentration and purification of the virions.

Purified concentrated inactivated TBE vaccine was reported to reduce considerably the incidence of TBE, particularly in Austria (Kunz et al., 1976, 1980; Barrett et al., 1999) (http://www4.tbe-info.com/vaccination/vaccination.html) and is now commercially available from two producers in Austria and Germany (Barrett et al., 1999).

In Austria, the vaccine was used extensively and although no controlled clinical trials were reported, it was considered to have performed efficiently on the basis of the high levels of seroconversion and the progressive reduction of numbers of cases of TBE. Since 1980, 35 million doses of vaccine have been used, 6.8 million people have been vaccinated in Austria and the estimated rate of protection is 96–99% in vaccinees that have completed the immunisation protocol of three doses. The vaccine is safe for use in both adults and children and established long-lasting immunological memory (Barrett et al., 1999). All rare recorded incidences of TBE among vaccinees have either been related to cases with an incomplete course of vaccination or have occurred in people over the age of 60 years (Barrett et al., 1999; Kunz, to be published). The increasing vaccination coverage resulted in a steady decline of the morbidity of TBE in Austria and in the last 3 years 41, 60 and 54 cases of TBE were recorded annually, in contrast with the neighbouring Czech Republic and Slovakia, where intensive vaccination programs were not applied. Here, over 700 TBE cases were registered annually for the last several years (M. Labuda, personal communication).

Overall, the Austrian experience has demonstrated that following intensive immunisation programs and a systematic approach, the disease can be almost eliminated (Kunz, to be published).

Nevertheless, Russia presents a very different problem because firstly, many residential areas are located amongst the forests and the risk of exposure to ticks is consequently very high. Secondly, TBEV strains circulating in Russia, belong to the Siberian and Far Eastern subtypes which, as described above, are more virulent than strains of the European subtype. Vaccine similar to that produced by the Austrian company "Immuno" is available in Russia. Laboratory experiments with mice showed the same level of protection as for the Austrian vaccines (Vorob'eva et al., 1996). In small human trials (600,000 people) the vaccine was found to be highly immunogenic and to have low reactogenicity (Chumakov et al., 1991), but its application has been limited due to logistical and financial constraints and, thus, its efficacy against Russian TBEV strains in large scale trials has been difficult to evaluate. Instead, a more cost-effective, non-concentrated tissue culture vaccine based on TBEV strain 205 is still in use in Russia. Since "Perestroika" the application of this vaccine has been reduced but even in the years when vaccine and pesticide controls were being carried out, there were still many cases of TBE registered annually even amongst vaccinated individuals (Leonova, 1997). Clinical surveys demonstrated longer incubation periods among vaccinated people compared with non-vaccinated, but there was no difference in the severity of disease between these two groups. Tick-borne encephalitis is therefore still a major problem in Russia requiring significant medical resources and research efforts.

Why did inactivated vaccines fail to afford absolute protection? The first possible reason is that different batches of commercial vaccines in Russia could vary in concentration of the active antigenic components and this is supported by the observed differences in efficiency of seroconversion in vaccinees (unpublished observations). Secondly, formalin inactivation could alter the antigenic properties of the surface protein by destroying some epitopes and the inactivated virus might then fail to induce antibodies against all the viral surface epitopes. This could provide the opportunity for virus to escape neutralisation and result in the development of the disease. The development of vaccine based on recombinant virus particles and non-structural virus proteins that does not require formalin inactivation might overcome this problem in the future (Heinz et al., 1995; Dmitriev et al., 1996; Holzer et al., 1999).

The third possibility could be the circulation of several virus strains that differ from vaccine virus in their antigenicity. Antigenically defective TBEV strains has been identified in Russia that very poorly react with commercial diagnostics (Pogodina et al., 1992). The development of a polyvalent vaccine using a mixture of different strains of TBEV might resolve this problem. However, this would require significant resources and a highly developed medical infrastructure.

Several new types of TBE vaccine are under development including the production of recombinant subviral particles that represent empty virus envelopes containing E and prM proteins (Heinz et al., 1995; Allison et al., 1999; Holzer et al., 1999). Naked plasmid DNA that expresses secreted subviral particles also induces immune protection against lethal doses of TBEV (Schmaljohn et al., 1997, 1999; Aberle et al., 1999). A protective role induced by the nonstructural proteins NS1, NS3 and NS5, that stimulate the development of T-cell immune responses, has also been demonstrated (Kulkarni et al., 1992; Gagnon et al., 1996; Zeng et al., 1996; Chen et al., 1999) and therefore including recombinant nonstructural proteins into the future synthetic vaccine might ensure better protective immunity.

12.2. Trials of live attenuated LGTV-based vaccine

Several strains of TBEV were tested in Russia as potential candidates for live attenuated vaccine. They were produced either by serial subculture of viruses or mutation using chemical agents. Several of these viruses passed safety tests in monkeys and human volunteers (reviewed by Pogodina et al., 1986; Smorodintsev and Dubov, 1986; Kamalov et al., 1989) but subsequent pathomorphological analysis showed evidence of persistent/chronic infection in the different organs and tissues of experimental animals (Pogodina et al., 1986) or rapid reversion to virulent phenotype after further passage (Kamalov et al., 1989).

In the 1970s attempts were undertaken to isolate attenuated variants of LGTV strain TP21 and one of them, Elantcev 15-20/3 was used to vaccinate two groups totalling 649,470 Russian volunteers. Meningoencephalitis with permanent neurological sequelae developed in a total of 35 patients (Leonova, 1997; Smorodintsev and Dubov, 1986; Zlobin and Gorin, 1996) with 32 cases in the first group of 379,540 individuals that had no preliminary vaccination and three cases in the second group of 269,939 people that had received a preliminary injection of inactivated vaccine. Although laboratory experiments with the candidate vaccine virus Elantcev 15-20/3 had not produced death in any of the monkeys, following intracerebral inoculation, 30% of the animals had developed neurological lesions from which they subsequently recovered (Smorodintsev and Dubov, 1986).

Nonetheless retrospective analysis on volunteers 20 years after the vaccination demonstrated several advantages of using live attenuated vaccine. Firstly, live vaccine reduced the incidence of TBE in endemic regions by more than 10 times in comparison with an inactivated vaccine, and by more than 20 times in comparison with a control group given a placebo (Shapoval et al., 1989). Secondly, a single immunisation with live vaccine produced long-lasting (for years) seroconversion in 100% of individuals whereas inactivated vaccine required multiple vaccinations to achieve a 90–100% immune response that in 25–40% of vaccinees lasted for 6 months. Thirdly, there was no increase in the incidence of the major human diseases (heart attacks, strokes, cancer, infections, etc.) or infectious diseases in the vaccinated group compared with control non-vaccinated individuals. In fact the lowest level of infectious diseases was registered in the group vaccinated with LGTV (Shapoval et al., 1989).

However, human trials with live attenuated vaccine based on LGTV have also revealed two major problems. The first is the high incidence of encephalitis 1:10,000; indicating that the strain chosen for the clinical trials was not suitable. Indeed, higher neurovirulence of Elantcev 15-20/3 in comparison with the original LGTV TP21 strain or other clones selected from the same virus population was confirmed later when more sensitive pathomorphological tests were developed (Kamalov et al., 1993; Sokolova et al., 1994a,b).

The second problem with these live attenuated vaccines is their failure to provide absolute protection against TBE infections in endemic regions. The first explanation could be the absence of appropriate antigenic cross-reactivity between LGTV and local TBEV strains that, in conjunction with the reduced levels of immunity fail to provide appropriate protection. *Langat virus* is a different species from TBEV, sharing only 82–88% of amino acid identity with TBEV, whereas within the TBEV group the lowest identity is 95–96% (Gritsun et al., 1993a,b). The second explanation and potential problem for live attenuated vaccine in Russia is the reported seronegative cases of TBE (Leonova et al., 1980; Pogodina et al., 1992). Currently, there is no satisfactory explanation for this, although immunological tolerance (Kobayashi, 1975; Cihak and Lehmann-Grube, 1978; Seto et al., 1988; Wohlsein et al., 1992; Mauracher et al., 1993; Kobets et al., 1995; Watts et al., 1999) and/or immunological suppression (Fugier Vivier et al., 1997; Oldstone, 1998; Sevilla et al., 2000) are just two possibilities. In fact, a proportion of people in endemic regions have not shown any immunological response following vaccination with inactivated or LGTV-based live vaccine (Pogodina et al., 1986). In this respect, the use of live attenuated virus as a vaccine could result in virus invasion into the CNS of seronegative people with unpredictable consequences.

New research has begun to explain many aspects of virus attenuation and may offer new methods to engineer custom-designed viruses with predictable pathogenicity. It has now been established that alteration of different virus functions through single point mutations in any gene or untranslated region has the potential to produce attenuation (Gritsun et al., 1990, 2001; Cecilia and Gould, 1991; Cahour et al., 1995; Chambers et al., 1995; Proutski et al., 1997a; Mandl et al., 1998; Xie et al., 1998; Amberg and Rice, 1999; Allison et al., 2001). Nevertheless, the sequences of the two most well recognised live attenuated flavivirus vaccines, *Yellow fever virus* (YFV) and *Japanese encephalitis virus* (JEV), have multiple substitutions over the entire virus genome in comparison with their wild-type parent viruses (Rice et al., 1985; Hahn et al., 1987; Xin et al., 1988; Aihara et al., 1991; Wills et al., 1993; dos Santos et al., 1995). It has been suggested that reliable attenuation of vaccine viruses can be achieved through the cooperative effect of many point mutations and experimental evidence now exists to confirm this. It was shown that five point-mutations that mapped in different regions of the TBEV genome, each produced a small biological change, that is not always noticeable in all biological tests, but the cumulative effect of these mutations led to attenuation of the virus (Gritsun et al., 2001). Equivalent findings were also reported for the mosquito-borne flavivirus, JEV (Arroyo et al., 2001). The advantage of attenuating a virus using a series of point mutations, each of which contributes only a small amount to attenuation, would be to reduce the risk of back mutations.

Several TBEV infectious clones have been constructed to study the different aspects of TBEV pathogenesis and to develop live attenuated vaccines (Mandl et al., 1997; Gritsun and Gould, 1998; Pletnev, 2001). Each of these clones could be used as a basis for further virus attenuation to produce vaccine strains.

Another approach for the development of live attenuated virus vaccines is based on the construction of chimaeric viruses using infectious clones of non-encephalitic mosquito-borne flaviviruses as a backbone, for example either the vaccine strain YF17D or Dengue type 4 virus. Two genes encoding the envelope glycoprotein and the prM protein have been substituted with the corresponding genes in the infectious clone (Pletnev et al., 1992; Monath et al., 1999, 2000). This strategy appears to have resulted in further attenuation of the viruses and might be suitable for the development of live attenuated vaccines for other flaviviruses. For TBEV vaccine a chimera was created using the Dengue 4 virus infectious clone and LGTV as a source of E and prM. The derived chimaeric virus was attenuated and protected animals in laboratory experiments against challenge with highly virulent TBE viruses (Pletnev et al., 2000, 2001).

In summary, despite the fact that the first large human trials with live attenuated vaccine against TBEV based on LGTV were unsuccessful, retrospective analysis among vaccinees revealed several advantages from the use of live attenuated vaccine in comparison with inactivated virus. These included superior seroconversion, more effective protection and the absence of chronic neuronal disease. For these reasons, the development of live attenuated vaccine, although not LGTV-based, still seems to be attractive as a preventive measure against TBEV. Currently there are no suitable strains of TBEV virus that could be used as attenuated vaccine candidates. Nevertheless, molecular–biological research is now being directed towards the development of reliable TBEV attenuation (Cahour et al., 1995; Mandl et al., 1998; Gritsun et al., 2001) that in future could be the basis of future genetically engineered vaccines.

13. Bioterrorism and TBEV

In the context of bioterrorism, we have shown that the tick-borne flaviviruses are pathogenic for humans and some animals. Some strains are more virulent than others but even the most virulent viruses are unlikely to produce high fatality rates. These viruses can infect via the alimentary tract and also when inoculated intranasally into experimental animals. Presumably, therefore concentrated aerosols would

be infectious or high virus concentrations delivered as a powder contaminating food might infect a significant proportion of people eating the food.

Tick-borne flaviviruses are excreted in the urine and faeces of experimentally infected animals but it is unlikely that this form of virus would provide an efficient route of infection for humans. Perhaps their greatest weakness as biological weapons is the fact that they are normally transmitted to vertebrate hosts via the bite of an infected tick, and the natural habitat of ticks is the forest or moist thick grassy vegetation as found on uplands. Under most circumstances this means that humans and even most animals would be a dead-end for virus transmission because few humans are exposed to the bite of a tick. Another important factor is that these viruses are all antigenically closely related. Therefore, immunity against one strain is likely to produce cross-immunity against the others. Moreover, in endemic regions there is a reasonably high level of immunity amongst the indigenous viruses.

One can ask the question whether or not it is feasible to spread the virus by infecting large numbers of ticks with the virus. This would not be a logical approach for the following reasons: (a) very large numbers of infected ticks would be required and logistically this would be technically extremely difficult; (b) ticks only feed three times, at very critical stages of their life cycle and it would be extremely difficult to arrange for them to be infected and ready to feed when delivered as weapons; (c) the production of a sufficiently large number of ticks to pose a threat to human or animal populations would also be a difficult technical exercise.

In summary, these viruses are unlikely to be the most effective front line weapons in biological warfare but they might be capable of causing significant problems on a small scale.

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