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Mini Review

A Mediterranean arbovirus: The Toscana virus

Marcello Valassina, Maria Grazia Cusi, and Pier Egisto Valensin

Department of Molecular Biology, Section of Virology, University of Siena, Siena, Italy

Toscana virus (Bunyaviridae family, Phlebovirus genus) is a sandfly fever virus responsible for human neurological infections. Sandfly viruses are transmitted by insect vectors (Phlebotomus species) and the infection is present in climatic areas that allow the life cycle of the vector. The arthropode-borne Toscana virus is the etiologic agent of meningitis, meningoencephalitis, and encephalitis. The frequency of this neuropathic infection increases in the summer months, peaking in August in the endemic Mediterranean areas (Italy, Portugal, Spain, and Cyprus). Infection diagnosis is carried out by molecular assays and immunoenzymatic tests, which are rapid and sensitive. Recent studies have investigated the antigenic properties of the viral proteins (nucleoprotein N and surface glycoproteins G1 and G2), to better understand their immunogentic role. *Journal of NeuroVirology* (2003) **9,** 577–583.

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Introduction

Many viruses are transmitted to man through arthropods, and many species of insects are involved in numerous geographical areas. Among the types of arbovirus transmitted to man, the Bunyaviridae family is large and it is composed of five genera: Bunyavirus, Hantavirus, Nairovirus, Phlebovirus (associated with the Uukuvirus), and Tospovirus which recently generated great interest in the botanic field (Beaty and Calisher, 1991). The Phlebovirus genus includes 37 recognized viruses, such as the sandfly fever viruses, and are mostly transmitted to vertebrates by phlebotomine, although other arthropods are also recognized vectors (Nicoletti and Varani, 1985). Phleboviruses are geographically distributed in Europe, Africa, Central Asia, and the Americas (Gonzales-Scarano *et al*, 1991; Touny *et al*, 1989). Little is known about the host animal reservoir: isolation tests and serological assays have suggested some animal species as candidates, although it was not possible to define their effective reservoir roles. It seems that the role of the animal in the survival of the virus is secondary to the horizontal amplification of the virus, which is, however, able to guarantee its transovaric and venereal transmission (Tesh, 1988; Tesh and Modi, 1987; Tesh *et al*, 1992; Maroli *et al*, 1993). Among the viruses belonging to the Phlebovirus genus, Rift Valley virus and sandfly fever virus are the most important (Meegan and Bailey, 1988; Tesh *et al*, 1976). In the latter group, there are some viruses circulating in Europe, which are responsible for acute nonfatal, influenza-like symptomatology (Eitrem *et al*, 1991b; Hertig and Sabin, 1964). Among these strains, in Europe there are three circulating serotypes: Sicilian virus, Naples virus, and Toscana virus. The Sicilian and Naples viruses are also present in Central Asia and the Middle East and they have the same vector distribution. The Toscana virus is present in Italy, Algeria, Spain, Portugal, and Cyprus, as noted in studies of the indigenous population and cases of infection in tourists visiting these areas (Ehrnst *et al*, 1985; Calisher *et al*, 1987; Endris and Perkins 1987; Eitrem *et al*, 1990, 1991; Schwarz *et al*, 1993; Dobler *et al*, 1997). Toscana virus was initially isolated from *Phlebotomus pernicious* in central Italy in 1971 and in 1980 it was registered in the *International Catalogue of Arbovirus*, and assigned to the Phlebovirus genus. Other strains of Toscana virus were isolated from *Phlebotomus perfiliewi* in other areas of Italy and from the brains of bats, *Pipistrellus kuhli*, captured in areas where the insect vectors were present (Verani *et al*, 1982, 1984a, 1988; Ciufolini *et al*, 1985). Toscana virus presents a distinct neurovirulence, a characteristic that it shares with the Rift Valley virus: they are the only neurovirulent viruses of the Phlebovirus group. Different from the Sicilian and Neaples viruses, which cause a febrile disease

Address correspondence to Prof. Pier Egisto Valensin, Department of Molecular Biology, Microbiology Section, University of Siena, Via Laterina, 8 53100 Siena, Italy. E-mail: valensinpe@unisi.it

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that lasts several days, Toscana virus infection is characterized by high fever, severe headache, aseptic meningitis with a benign course, followed by a medium-long convalescence (Nicoletti *et al*, 1991, 1996; Braito *et al*, 1998a, 1998b). Occasionally there is an extension of the encephalic infection, creating cases of meningoencephalitis or encephalitis, sometimes without meningitis (Dionisio *et al*, 2001). Its neurovirulence was demonstrated in pathogenicity studies carried out *in vivo* and in studies where the virus was isolated from cerebrospinal fluid from patients with meningitis, in which serological analysis confirmed the etiological role of the virus.

Molecular and biological characteristics

Viruses belonging to the Phlebovirus genus present molecular and biological characteristics of the Bunyaviridae family: the presence of a negative-strand RNA genome, represented by three segments, S, M, and L (small, medium, and large), the presence of an envelope with the G1 and G2 glycoproteins, the absence of an M protein (as opposed to other enveloped riboviruses), and the presence of a one-step maturation at the level of the Golgi apparatus in the host cell (Bishop 1990; Schmaljohn 1996).

The viral particles have a diameter of 80 to 120 nm and are composed of helicoidal nucleocapsids containing the trisegmented genome associated to the nucleoprotein (coded by the S segment) and to the viral polymerase (coded by the L segment). The genomic segments have short genus-specific sequences at the 3′ end complementary to the 5′ end, which are responsible for the formation of panhandle structures that are probably associated with one another. These structures seem to be important for transcription mechanisms and genome replication, or for genome packaging and for viron assembly. The segments associated with numerous copies of the N nucleoprotein are included in helicoidal nucleocapsids in the virions in a non-equimolar ratio (Elliott *et al*, 1991; Pringle, 1991).

The L segment (6400 to 6700 nucleotides [nt] in the Phleboviruses) contains a single open reading frame (ORF) that encodes the L protein. The protein of 2095 amino acids in the Toscana virus (239 kDa) presents strong homology in the central portion with the Rift Valley virus. The L ORF is expressed via a viral-complementary mRNA. In this regard, the organization of the Toscana virus L segment could be different from the L segment of Bunyamvera (Elliott, 1989) and Rift Valley (Elliott *et al*, 1991) viruses in which a short ORF was found in the 3′ region of the antigenome, even though there was no evidence for its expression. Comparing the deduced amino acid sequences of Toscana virus L protein to the counterpart sequence of other negative-strand RNA viruses identified a marked homology with the Rift Valley virus L protein. The overall homology of the two proteins is 37%, but is not uniformly distributed. The more conserved region lies in the central part of the molecule, spanning amino acids 650 to 1600 (68% overall homology). This kind of conservation could indicate that the central region of the protein represents the functional domain, whereas the differences in the terminal regions might reflect species divergence. Comparison of Toscana virus L protein and the available sequences of L polymerases of other negative-strand viruses indicated no conservation in members of the same family. On the contrary, the strong homology of Toscana virus and Rift Valley virus L proteins, together with the similarities in the coding strategy between their N proteins, suggest that these viruses are strongly related in their evolution (Elliott *et al*, 1991; Accardi *et al*, 1993, 2001).

For the Toscana virus, a gene product of 1339 amino acids has been proposed for the M segment (4215 nt), with a molecular weight of around 149 kDa and nine sites of glycosylation. The single ORF of Toscana virus M segment encodes three proteins, Nsm (30 kDa) and the two glycoproteins G1 and G2 (Gn and Gc, respectively, according to the nomenclature proposed by Lappin *et al* [1994]) of the same molecular mass (65 kDa), with a genomic order NH_2-NSm-G1-G2-COOH with respect to the putative precursor. The major regions of homology with the corresponding sequence of other phleboviruses were found in the carboxy half of the M gene product. The hydropathy profile of the Toscana virus M precursor protein reveals a striking similarity to those of other phleboviruses, suggesting a common transmembrane topology. It has been concluded that the amino terminus of each glycoprotein is preceded by a stretch of hydrophobic amino acids. By alignment of the Toscana virus M sequence with corresponding sequences of Rift Valley virus and Punta Tora virus, it has tentatively placed the start of Toscana virus G1 and G2 glycoproteins at residues 297 and 936 of M ORF, respectively. As in the other genomic segments, the M segment appears to be related to those of other phleboviruses, except for the region coding for the nonstructure proteins NSs. These proteins are the most variable proteins among the phleboviruses, and they could play a very specific role in each virus. G1 and G2 glycoproteins seem to be responsible for the host virus-cell interactions (Elliott *et al*, 1991; Lappin *et al*, 1994; Di Bonito *et al*, 1997; Grò *et al*, 1997).

Finally, the S fragment (1869 nt) codes for the N nucleoprotein (27 kDa) and the nonstructural NS protein (37 kDa), through a particular transcription and translation process defined as "ambisense." A viral complementary, subgenomic mRNA corresponding to the 3′ half of the viral S RNA codes for the N protein, and the viral sense subgenomic mRNA corresponding to the 5′ half of the viral S RNA codes for the NS proteins. In the region between the two ORFs, among the Phleboviruses, there is a short intergenic region with numerous C homopolymers adjacent to conserved GCTGCC hexanucleotides. The Toscana virus N and NS mRNAs cover the intergenic region and overlap by about 80 residues. Because the

mRNAs are not encapsidated, their complementary 3′ terminals could facilitate their *in vivo* annealing. This could be important in the control of N and NS mRNAs stability (Elliott *et al*, 1991; Giorgi *et al*, 1991; Grò *et al*, 1992). The immune response to the nucleoprotein is strong and it could be involved in some protection mechanisms that have been demonstrated *in vitro* (Cusi *et al*, 2001). Some variations in the central region of the S segment sequence have shown the first evidence of some circulating variants of the Toscana virus (Valassina *et al*, 1998a) and the significance of these has yet to be defined.

Antigenic characteristics

Among Bunyaviridae, antigen variability is due to mutations and reassortment of genome segments (Kingsford, 1991). The antigen characteristics of Bunyaviridae can be analyzed through serological tests. Hemoagglutination inhibition and neutralization tests allow us to find closely related viruses on the basis of the surface glycoproteins belonging to the same serogroup. Viruses that belong to a species or different serotypes within the same serogroup can be recognized by analyzing the antigen relationships between the nucleocapsid proteins. The recognition of subtypes facilitates identification of minor differences that can determine the presence of serotypes or strains. For Phleboviruses, through the use of monoclonal antibodies, it was possible to identify, for the Punta Toro virus, neutralizing and hemoagglutinating epitopes on the G1 protein and at least three neutralizing sites on the G2 protein (Pifat *et al*, 1988). Studies carried out on the Rift Valley virus, responsible for hemorrhagic fever in the subtropical areas of Africa, have shown the presence of neutralizing epitopes on surface glycoproteins (Saluzzo *et al*, 1989a). One study of monoclonal antibodies against the nucleoprotein also showed the presence of two strains circulating in Egypt and Mauritania, originating from a common ancestral precursor, suggesting the possible influence of ecological factors that could have favored epidemic diffusion (Saluzzo *et al*, 1989b).

The nucleoprotein of the Toscana virus is characterized by a strong degree of immunogenicity as shown by enzyme-linked immunosorbent assay (ELISA) and Western blot tested on human sera positive for Toscana virus (Schwarz *et al*, 1996, 1998; Magurano and Nicoletti, 1999). The antibody response is long-lasting and appears to be partially protective by evaluating the inhibition of the formation of virus plaques with mouse serum inoculated with the recombinant nucleoprotein (Cusi *et al*, 2001). Immunoblotting and semiquantitative radioimmunoprecipitation assay (RIPA) allow the identification of nucleoprotein N as the major antigen responsible for both immunoglobulin M (IgM) and IgG responses. Antibodies to proteins other than N protein are detected only by RIPA. Antibodies to glycoproteins are detected in about one third of the patients, and because their presence always predicts neutralization, some serum samples with neutralizing activity have undetectable levels of antibodies to G1-G2 (Di Bonito *et al*, 1999). These results raise some questions about antigenic variability and relevant neutralization epitopes of Toscana virus. The specific antibody response on the epitopes of the surface glycoprotein has been analyzed only recently by using Toscana virus recombinant proteins expressed in baculovirus (Di Bonito *et al*, 2002). In this system, all the sera reacted with the N protein, but they differed in their response to the glycoproteins. This is probably due to the technical limitations of the conformational maintenance of the epitopes of the recombinant glycoproteins. However, the anti-glycoprotein antibody response does not appear to be homogeneous among infected persons.

Diagnosis

The clinical manifestation of meningitis from Toscana virus is not different from that caused by other viral agents. Therefore, it is not possible to define a characteristic symptomatology for neurological infections from Toscana virus. As for the other types of aseptic meningitis, an effective and rapid diagnostic approach, aimed at a rapid recognition of the viral agent, is necessary in order to exclude bacterial agents for which antibiotic therapy is effective (Rotbart, 1997). The clinical course of infection, as in other forms of aseptic meningitis, is generally benign, with less frequent encephalic involvement. Direct diagnosis of acute viral neurological infections is known to be difficult, due to the rapid viremic phase and the presence of a low viral load at the time of clinical symptomatology, often corresponding to the moment of hospitalization and clinical sampling. Toscana virus can be isolated *in vitro* on Vero cells (Verani *et al*, 1984a, 1984b). The growth of the virus causes a lytic cytopathic effect. The hemoagglutinating activity of the envelope glycoproteins is revealed with goose red blood cells by the hemoagglutination test. Isolation of the virus can also be carried out by intracerebral inoculation in mice. Notable diagnostic success in Toscana virus infections was achieved through molecular reverse transcription–polymerase chain reaction (RT-PCR) techniques, selecting primers on the S fragment (Valassina *et al*, 1996, 2000). This approach has shown the presence of different Toscana virus variants circulating in Tuscany during different years (Valassina *et al*, 1998b). Moreover, the setting of multiplex PCR methods has allowed the identification, at the same time, of other neurotropic viruses (particularly enteroviruses), which circulate during the same season, thus reducing time, expense, and the risk of sample manipulation (Valassina *et al*, 2002).

Indirect diagnosis is also useful by assaying for the specific IgM as a marker of acute infection. The use of immunoenzymatic techniques has been very useful in the preparation of tests in which the recombinant

nucleoprotein expressed in *Escherichia coli* is used as the specific antigen, allowing the exclusion of cross-reactive phenomenon with the other two circulating serotypes, Naples and Sicilian (Valassina *et al*, 1998a; Soldateschi *et al*, 1999; Ciufolini *et al*, 1999). Immunofluorescence is a useful alternative, using Vero cells infected with Toscana virus (Mendoza-Montero *et al*, 1997; Schwarz and Jager, 1995). Techniques such as complement fixation, and above all, neutralizing assays, such as the reduction of the formation of plaques and the inhibition of cytopathic and/or hemoagglutinating effects, are methodologically more complex and less suitable. However, the plaque-reduction test is the most common assay for assessing the antibody titre, allowing a correct evaluation of the neutralizing activity of the specific antibodies. The Western blot assay is less reliable for detection of anti-G1 and anti-G2 antibodies, perhaps due to a technical limitation that does not allow the conformational maintenance of the epitopes involved in the humoral response (Di Bonito *et al*, 1999, 2002; Magurano and Nicoletti, 1999).

Epidemiology

Phlebotomus fever viruses are transmitted to humans by phlebotomus flies and produce an acute, nonfatal, influenza-like illness. The vectors are members of the Phlebotomus, Sergentomyia, and Lutzomya genera. The description of an acute illness that probably represented phlebotomus fever dates back to the time of Napoleonic wars and was reported as Mediterranean fever. The interest in phlebotomus fever increased with the epidemics of the disease in Allied troops in Italy during World War II in 1943–1944. It was demonstrated that the disease was caused by two antigenetically distinct viruses (Sicilian and Naples) by intracerebrally inoculating suckling mice with acute-phase serum drawn from these soldiers. Cross-challenge experiments in human volunteers with Sicilian and Naples viruses showed that a single infection provided protection only against the homologous virus (Sabin *et al*, 1944). Toscana virus, the third member of the Phlebotomus fever serogroup of arboviruses, was isolated more recently, in 1971, from the sandfly *Phlebotomus perniciosus* in Italy. Toscana virus multiplied to high titres with a cytopathic effect in several vertebrate cell cultures (e.g., Vero, BHK-21, etc.), whereas it failed to replicate in mosquito cell cultures (Verani *et al*, 1982, 1984a, 1984b). It was shown to be closely related to Naples sandfly fever virus. The antigenic relationship between the two viruses was analyzed using complement fixation, plaque-reduction neutralization, and indirect fluorescent antibody tests (Gonzales-Scarano and Nathanson, 1990).

A serosurvey for the presence of antibodies to Sicilian sandfly fever, Naples sandfly fever, and Toscana viruses indicated that, as in other Mediterranean areas, both Sicilian sandfly fever and Naples sandfly fever viral infections decreased or disappeared after the 1940s in countries with insecticide-spraying malaria eradication campaigns, whereas Toscana virus was still observed annually in Central Italy during the summer, causing aseptic meningitis or meningoencephalitis. These data suggest the hypothesis of the presence of an unknown animal reservoir, which could be implied in the maintenance of the Toscana virus. Epidemiology studies have demonstrated a high incidence of meningitis from Toscana virus during the summer months, with a peak during the month of August, corresponding to the maximum activity of the vector. The target population generally comes from geographical areas where Toscana virus is not endemic, mostly tourists who are visiting the endemic area. Meningitis cases are less frequent among individuals from endemic zones, probably due to the prevalence of anti–Toscana virus antibodies present in the resident adult population (20%) (Tesh *et al*, 1976; Nicoletti *et al*, 1991, 1996; Magurano and Nicoletti, 1999; Schwarz and Jager, 1995).

Toscana virus is present in other Mediterranean countries where Phlebotomus genus sandflies are present. The few epidemiological data available mostly refer to Central Italy where cases of aseptic meningitis are more frequent during the summer. Sporadic cases of menigitis have been reported in tourists travelling in the endemic areas of the Mediterranean (Ehrnst *et al*, 1985; Calisher *et al*, 1987; Schwarz *et al*, 1993; Dobler *et al*, 1997). A recent study performed on the etiology of meningitis in Tuscany (Valassina *et al*, 2000) demonstrated that the Toscana virus has an incisive role and that it is responsible for 81% of cases of aseptic meningitis that occurred during the summer. More recently, thanks to PCR and more specific recombinant immunoenzimatic assays, research of Toscana virus as an etiologic agent of neurological diseases has been carried out in other regions of Northern Italy (Emilia Romagna and Piedmont) and in the central region of Spain (Portolani *et al*, 2002; Echevarria *et al*, 2003). Serological analyses also showed the presence of asymptomatic infections of Toscana virus. A serological investigation carried out on 83 asymptomatic household contacts of 46 central nervous system summertime infection patients showed an anti–Toscana virus IgG seropositivity in 22% of the subjects and IgG/IgM seropositivity in 6% of the subjects (Braito *et al*, 1997, 1998). A recent study including 360 subjects of a high-risk, professionally exposed population, reported a seropositivity in 70% of subjects without neurological symptomatology (Valassina *et al*, 2003). This confirmed that Toscana virus infection can occur with either mild or no symptoms. These data show the frequent presence of the Toscana virus infection in the regions where the specific vector lives. The serological data have been very useful for epidemiological observation, allowing the evaluation of seropositivity in endemic populations and the study of the possible circulation of Toscana virus in other geographic areas (Portolani *et al*, 2002; Echevarria *et al*, 2003).

Conclusions

Toscana virus is an interesting example of arbovirus circulating in the Mediterranean area, the diagnosis of which has been facilitated by the introduction of new molecular methods and by the introduction of immunoenzymatic tests based on the use of the recombinant nucleoprotein. Although diagnostic improvement has permitted the recognition of the Toscana virus as the main etiologic agent of summer meningitis in Italy, further studies concerning the knowledge of the pathogenic mechanisms and of its etiologic role in less suspected geographical areas (central Spain) are necessary. Gene analysis has allowed evaluation of the presence of mutations at the level of the S fragment, with the possible identification of circulating variants, possibly responsible for neurologic and/or asymptomatic infections. Further analysis of the envelope glycoproteins, directly involved in the mechanisms of virus-cell interaction and in the immune response of the host, would be useful. Preliminary studies have shown a partial protective role of the anti-N antibodies, *in vitro* and *in vivo*. However, the effective protective role of the anti-G1 and -G2 antibodies is not as yet clear. New studies are in progress, regarding the cytotoxic response, in order to explain the exact role of the cell-mediated immune response of symptomatic and asymptomatic patients in protection against the disease. Preliminary studies, carried out on mice, have shown that the cytotoxic response to the N protein is predominant. Further studies are necessary to create a valid animal model for Toscana virus that could elucidate the pathogenetic mechanisms of Toscana virus *in vivo*, and the role of the viral structural proteins in the immune response. This information would therefore be the basis for the preparation of a potential recombinant vaccine that would be useful for combating this benign, yet worrisome disease, particularly for the tourist population coming from nonendemic areas.

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