Acta virol. **28**: 39—47, 1984

ANTIGENIC AND BIOLOGICAL CHARACTERIZATION OF TOSCANA VIRUS, A NEW PHLEBOTOMUS FEVER GROUP VIRUS ISOLATED IN ITALY

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Received May 18, 1983

Summary. — Toscana (TOS) virus, a new member of the Phlebotomus fever serogroup of arboviruses, was isolated from sandflies collected in Italy. It was shown to be closely related to Sandfly fever Naples (SFN) virus. The antigenic relationships between the two viruses were analysed in the complement fixation (CF), plaque reduction neutralization (PRN) and indirect fluorescent antibody (IFA) tests. TOS virus multiplied tc high titres with a cytopathic effect (CPE) in several vertebrate cell cultures (e.g. Vero, BHK-21, etc.), whereas it failed to replicate in mosquito cell cultures. Plaque formation by TOS virus in Vero cells was not affected by incorporating dimethyl sulphoxide in the overlay, which is known to enhance plaque formation by SFN virus. Some biological characteristics in cell cultures and in mice of a mouse-adapted and a cell-adapted TOS virus strain were studied. After 4—6 passages in the two different systems, changes in plaque size, in the amount of autointerfering activity and of virulence for mice were observed.

Key words: Toscana virus; *Bunyaviridae*; *Phlebotomus fever group*; *biological properties*; *strain differences*

Introduction

During field studies of arboviruses in Central Italy, three strains of a single virus were isolated from the sandfly *Phlebotomus perniciosus*. In a previous report, the circumstances of isolation and the serological relationships of ghis previously undescribed virus with members of the Phlebotomus fever troup of arboviruses were described (Verani *et al.*, 1980). Subsequently this virus was named "Toscana" (TOS) after the name of the region where the virus was first isolated, and was registered in the International Catalogue of Arboviruses as a new member, of the Phlebotomus fever serogroup (N. Karabatsos, Center for Disease Control, Ft. Collins, Colorado, personal communication, 1980).

The Phlebotomus fever group of arboviruses (family *Bunyaviridae*, genus *Phlebovirus*) is presently composed of at least 31 distinct virus serotypes

occurring in both the Old and New Worlds (Tesh *et al.*, 1982). Detailed investigation of biological and other characteristics of members of Phlebotomus fever serogroup has been in many cases hampered by their low virulence for animals and by the lack of a sensitive and reproducible assay system in tissue culture. TOS virus was lethal to newborn mice on initial passage and by brain-to-brain passages a mouse-adapted strain was readily established. Furthermore, TOS virus was also isolated in Vero cells from the original sandfly suspension and a cell-adapted strain was also established (Verani *et al.*, 1980). With the aim to better characterize the new *Phlebovirus* isolate, further studies were conducted to determine antigenic relationships between TOS virus and two other *Phleboviruses* (Sandfly fever viruses) isolated in Italy as well as the growth kinetics and other biological properties of the new virus. The results of these studies are described in this paper.

Materials and Methods

Viruses. Mouse-adapted TOS virus strain (TOS-MB) was used as a 10% brain suspension in the 4—6th mouse passage. Cell-adapted TOS virus strain (TOS-V) was used as culture fluid in the 4th Vero cells passage. Sandfly fever Naples and Sicilian (SFN, SFS) viruses were prepared from infected suckling mouse brains.

Cells. Vero, BHK-21, CV-1, RD and LLC-MK-2 cell cultures were grown at 37 °C in Minimal Essential Medium (MEM) supplemented with 10% foetal calf serum (FCS). Maitenance medium was MEM with 2% FCS. *Aedes albopictus* and *A. aegypti* cell cultures (from ATCC) were grown at 28 °C in Mitsuhashi-Maramorosh medium supplemented with 20% FCS.

Plaque assay. The plaquing procedure employed confluent monolayers of Vero cells and an overlay containing 1% of tragacanth gum, as previously described (Verani *et al.*, 1980). When appropriated, dimethyl sulphoxide (DMSO) and/or heparin were added to the standard overlay at the concentration reported by McCown *et al.* (1979).

Serological methods. CF and PRN tests were performed as previously described (Verani *et al.*, 1980). In the short-incubation PRN test, the prewarmed stock virus diluted to contain approximately 2×10^6 PFU/ml and the immune reagents diluted 1 : 10 were mixed and incubated at 37 °C. At the appropriate times, 0.1 ml of the mixture was removed, immediately diluted in 100 ml of cold diluent and titrated by plaque assay. For the IFA test, monolayers of Vero cells grown on 8-chamber slides (Lab-Tek) and fixed in acetone 24 hr post infection (p.i.) (multiplicity of infection—m.o.i.—1 PFU/cell) were used as antigen. Serial twofold dilutions of immune reagents were added to the appropriate wells which after incubation at 37 °C for 30 min were stained with goat anti-mouse or anti-rabbit immunoglobulins (Nordic).

Growth curves. Monolayers of Vero cells were infected with stock TOS-MB and TOS-V strains at different m.o.i. At selected time intervals, the culture fluids were collected, cells scraped of and stored at −70 °C until titrated by plaque assay.

Viral interference assay was performed by the method described by Kowal and Stollar (1980) with minor modifications. Briefly, duplicate monolayers of Vero cells were infected with standard plaque-purified TOS virus alone (1 PFU/cell) as a control or with a mixture of standard virus and twofold dilutions of either TOS-MB or TOS-V stocks. After 48 hr, culture fluids were collected and titrated by plaque assay.

Sensitivity to pH, temperature and lipid solvents. Acid sensitivity was determined as described by Shope and Sather (1979). For defining temperature stability, stock TOS-MB virus was diluted 1 : 10 in MEM containing 10% heat-inactivated FCS and aliquots of the dilution were stored at 56°, 37°, 28° or 4 °C. At selected time intervals, samples were removed and frozen at −70 °C. All samples were simultaneously titrated. Sodium deoxycholate and ether sensitivities were determined as previously described (Verani *et al.*, 1980).

Inoculation of mice. One litter of outbred Swiss mice was used for each virus dilution, with each suckling mouse receiving 0.01 ml intracerebrally (i.c.) or 0.02 ml intraperitoneally (i.p.),

Table 1. Results of complement fixation (CF), plaque reduction neutralization (PRN) an indirect fluorescent antibody (IFA) tests with SNF and TOS viruses

Ascitic fluid	Antigen/virus					
	SFN			TOS		
	CF	PRN	IFA	CF	PRN	IFA
SFN	**256/128**[a]	**160**[b]	**640**[c]	128/256	<10	80
TOS	64/64	40	160	**256/128**	**640**	**320**

[a]reciprocal of the highest ascitic fluid dilution/the highest antigen dilution
[b]reciprocal of the highest ascitic fluid dilution giving ≧80% plaque reduction
[c]reciprocal of highest ascitic fluid dilution (positive reaction)

each 14- or 28-day -old mouse receiving 0.03 ml virus suspension i.c. or 0.05 i.p. Average lethal time (ALT; sum of days between inoculation and death of all dead mice divided by sum of all dead mice) of mice inoculated with the two dilutions above and below 100 LD_{50} was calculated.

Results

Serologic relationships of Toscana and Sandfly fever viruses

Table 1 compares the results of CF, PRN and IFA tests with SFN and TOS viruses. By CF test, the two agents were rather similar, but by PRN technique they were antigenically distinct. Broad cross-reactivity between TOS and SFN viruses was also shown by IFA test. The reactions between SFS and both TOS and SFN viruses were quite specific by each of the three tests used (data not shown). The antigenic difference between TOS and SFN viruses was further confirmed by short-incubation neutralization tests. As shown in Table 2, both viruses were efficiently neutralized by homologous ascitic fluid after 10, 20 or 40 min of incubation, whereas only partial neutralization was observed with heterologous ascitic fluids.

Table 2. Results of short-incubation PRN tests with SFN and TOS viruses

Ascites	Virus	Ln Vt_{10}/V_0	Ln Vt_{20}/V_0	Ln Vt_{40}/V_0
TOS	TOS	1.11 (67.2)	1.49 (77.6)	1.23 (70.8)
	SFN	0.34 (29.3)	0.28 (24.6)	0.55 (42.6)
SFN	TOS	0.29 (25.7)	0.40 (33.4)	0.66 (48.5)
	SFN	1.60 (79.9)	2.14 (88.2)	3.04 (95.3)
Not immunized mouse	TOS	n. t.	n. t.	0.20 (18.1)
	SFN	n. t.	n. t.	0.04 (4.0)

* Natural log of the ratio of the virus titre at time 10 min (Vt_{10}) divided by the virus titre at the beginning of the test (V_0). In parenthesis percentage of reduction; n. t. = not tested.

Table 3. Effect of dimethyl-sulphoxide (DMSO) and/or heparin on TOS and SFN viruses (plaque assay in Vero cells)

Virus	DMSO	Heparin	Titre	Plaque diameter
TOS-MB	0	0	1.45×10^9	pinpoint
	0	10 μg/ml	1.00×10^9	pinpoint
	1%	0	1.95×10^9	pinpoint
	1%	10 μg/ml	1.27×10^9	pinpoint
SFN	0	0	2.55×00^7	+
	0	10 μg/ml	1.00×10^6	+
	1%	0	1.31×10^8	2 mm
	1%	10 μg/ml	5.50×10^7	+

+ difficult to observe and count

Growth in cell cultures

TOS-MB and TOS-V strains infected various vertebrate cell cultures producing CPE. The effect appeared regularly from day 2 p.i. and was complete between day 3 and day 4 in Vero, BHK-21 and CV-1 cells, whereas a high degree of cell degeneration was observed 6—7 days p.i. in RD and LLC-MK-2 cell cultures. The infectivity titres in Vero cells of TOS-MB strain ranged from 7.5 to 9.0 $\log_{10}$ PFU per g of brain, whereas those of

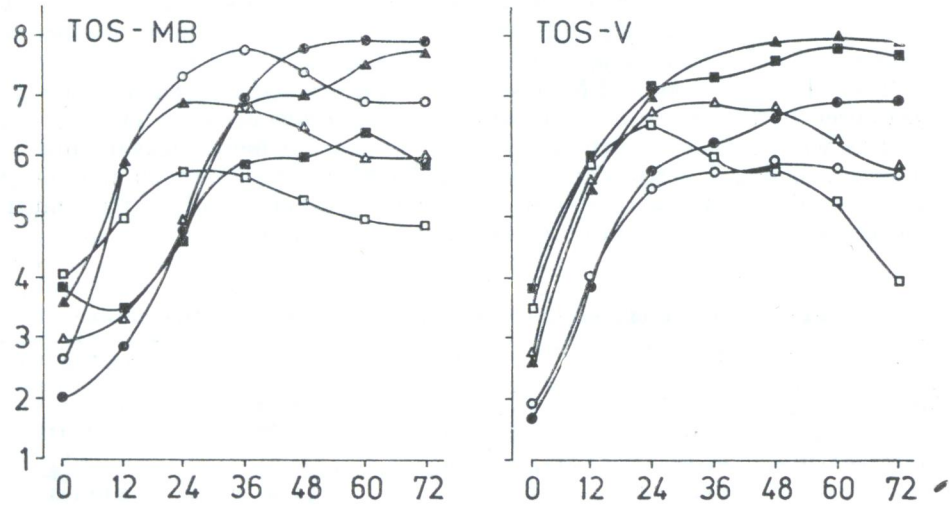

Fig. 1.

Growth curves of mouse-adapted and cell-adapted TOS virus as a function of input m.o.i. Abscissae: hr p.i. Ordinates: log PFU/ml
m.o.i. 0.01 PFU/cell: harvests from culture fluid (●—●) and from cells (○—○)
m.o.i. 0.1 PFU/cell: harvests from culture fluid (▲—▲) and from cells (△—△)
m.o.i. 1 PFU/cell: harvests from culture fluid (■—■) and from cells (□—□)

Table 4. Interference-induced reduction of the infectious yield assay

Interfering sample	m.o.i.	Titre (log_{10} PFU/ml)[a]	Yield difference (log_{10} PFU)
TOS-MB	2	3.27 ± 0.01	−1.28[b]
	1	3.03 ± 0.02	−1.52[b]
	0.5	3.06 ± 0.05	−1.49[b]
	0.25	3.25 ± 0.01	−1.30[b]
TOS-V	1	5.69 ± 0.06	+1.14[c]
	0.5	5.81 ± 0.07	+1.26[c]
	0.25	5.67 ± 0.02	+1.12[b]
	0.125	5.74 ± 0.00	+1.19[b]
None		4.55 ± 0.11	

[a]mean ± standard error of the mean
[b]$P < 0.01$
[c]$P < 0.05$

TOS-V strain were slightly lower, 7.0—7.5 log_{10} PFU per ml. There was no evidence of virus growth in *Aedes aegypti* and *A. albopictus* cell lines infected with TOS-MB virus. There was some maintenance of virus titre in both cell lines (1.87 log) by 10—14 days after inoculation, whereas the virus in the controls (virus and growth medium only) disappeared by the same period. Viral antigens were observed in the cytoplasm of a few cells stained by immunofluorescence 3 and 7 days after infection.

Plaque formation

TOS-MB virus readily formed clear, pinpoint plaques (0.4—0.6 mm in diameter) in Vero cell monolayers overlaid either with agar or with tragacanth gum after 5—7 days incubation. TOS-MB virus grown for one or more passages in Vero cells frequently yielded mixed population of plaques ranging from pinpoint to 2 mm in diameter. Single plaque purification failed to separate the stock containing the large plaque variant only since mixed plaque composition was found even after one further passage in Vero cells. When TOS-V virus was plated in Vero cells, plaques with a 2 mm diameter were always formed. Plaque size was found to remain stable either through 2—3 further passages or after plaquing isolated plaques for three serial passages. Immune ascitic fluid to TOS-MB virus neutralized to titre both strains.

The effect of adding DMSO and/or heparin was further tested on the size, number and clarity of plaques formed by TOS-MB virus. As shown in Table 3, there was no difference in plaque size or number regardless of DMSO addition into the overlay, whereas the known enhancing effect of DMSO on plaque formation by SFN virus was confirmed in a parallel test. Plaques produced by SFN virus without DMSO were barely visible and many assays yielded no visible plaques at all. The inclusion of heparin either alone or combined with DMSO did not affect plaque formation by either virus.

Table 5. Thermal stability of TOS-MB virus at various temperatures in relation to time of incubation

Time of incubation	Residual infectivity (log_{10} PFU/ml) at			
	4 °C	28 °C	37 °C	56 °C
0 min	6.3	6.3	6.3	6.3
15 min	n. t.	n. t.	n. t.	2.5
30 min	n. t.	n. t.	6.0	1.5
45 min	n. t.	n. t.	n. t.	<1
60 min	6.4	6.0	5.9	<1
120 min	n. t.	6.0	5.8	<1
10 h	5.9	5.7	5.5	n. t.
24 h	5.9	5.6	4.4	n. t.
48 h	5.9	5.4	2.2	n. t.
72 h	5.9	5.0	1.3	n. t.
7 days	5.8	4.3	<1	n. t.

n. t. = not tested

Kinetics of replication

Growth curves of TOS-MB and TOS-V strains in Vero cells as a function of the input m.o.i. are shown in Fig. 1. The titres of cell-associated virus in cells infected with either strain reached the maximum levels by 24 to 36 hr p.i., slightly declining thereafter. Peak titres of released virus were reached after 48—60 hr p.i. By this time, there were severe cytopathic changes in all cells and no further virus production.

Following infection of cells with TOS-MB strain, the highest infectivity titres of both cell-associated and released virus were at the lowest m.o.i. tested (0.01 PFU/cell), declining along with 10- and 100-fold increases in m.o.i. In contrast, infection of cells with TOS-V strain resulted in an increase in infectious virus production over the same time period along with increasing input m.o.i.

In cultures infected with TOS-MB strain, however, the majority of cells contained viral antigens, as measured by immunofluorescence, at the time when the production of cell-associated virus reached the maximum levels, regardless of input m.o.i. (data not shown). The proportion of antigen--positive cells increased to nearly 100% by 18 hr p.i. (m.o.i. 1 PFU/cell) and by 24 and 30 hr p.i. at m.o.i. of 0.1 and 0.01 PFU/cell respectively. These findings could be explained by the presence of auto-interfering particles in TOS-MB viral stocks. Experiments were therefore carried out to test the interfering activity in both TOS-MB and TOS-V preparations.

The data obtained in a representative experiment are shown in Table 4. When cultures were coinfected with standard virus and serial twofold dilutions of TOS-MB virus, a 95% reduction in infectious yield was observed in comparison with the standard virus alone. The extent of interference did not vary significantly in the range of dilutions tested. In contrast, when TOS-V virus was used as an interfering preparation, the total infectious

Table 6. Infectivity titres and average lethal times (ALT) in mice inoculated with mouse-adapted and cell-adapted TOS virus

Virus	Route of inoculation	Age of mice					
		1 day		14 days		28 days	
		Titre	ALT ± s.e.	Titre	ALT ± s.e.	Titre	ALT ± s.e.
TOS-MB	i. c.	8.5*	5.2 ± 0.2	9.0	4.8 ± 0.1	8.9	6.7 ± 0.1
	i. p.	6.2	7.1 ± 0.4	4.6	7.4 ± 1.3	<1	—
TOS-V	i. c.	7.4	8.9 ± 0.4	4.1	7.0 ± 0.4	3.3	8.6 ± 0.5
	i. p.	5.9	10.1 ± 0.4	<1	—	n. t.**	—

* $\log_{10}$ LD_{50}/g of brain or ml
**not tested
s. e. = standard error

yields were always higher than those obtained with the standard virus alone, as a consequence of increased multiplicity of infectious virus in the absence of interfering activity.

Effect of chemical and physical agents

Toscana virus was sensitive to the action of the lipid solvents sodium deoxycholate and diethyl ether; at least 4.1 and 3.4 log of virus, respectively, were lost after treatment. Infectivity of the virus exposed for 3 hr to pH 3 was almost abolished (titre < 3 $\log_{10}$ PFU/ml), whereas a control sample incubated at pH 7.5 titrated 8.9 $\log_{10}$ PFU/ml. Thermostability studies (Table 5) indicated that TOS virus was relatively stable at 4 °C for at least a week, but was less stable at higher temperatures. Growth of the virus was not affected by changes in incubation temperature. No significant differences in PFU titres were observed after incubation of infected monolayers at 33°, 37° or 39°. In order to investigate the possible presence of temperature-sensitive mutants in both TOS-MB and TOS-V stocks, the 2nd day yields of Vero cells infected with both strains and incubated at 33°, 37°, 39° or 41 °C were titrated at 37 °C. No differences greater than 0.2 and 0.5 $\log_{10}$ were observed in the infectivity titres of TOS-MB and TOS-V virus yields respectively at all temperatures tested.

Mouse inoculations

The results of inoculation of both TOS-MB and TOS-V strains in mice of different ages and by different routes are summarized in Table 6. I.c. inoculations of both strains caused death of newborn, 14-day- and 28-day-old mice. The infectivity titres of TOS-MB strain were consistently high in all age groups, whereas those of TOS-V strain were much lower in 14- and 28-day-old mice than in newborns. By i.p. inoculation, TOS-MB strain was lethal for newborn and 14-day-old mice, whereas TOS-V strain killed only

newborn mice. The ALTs of mice inoculated by any route with TOS-MB strain were always lower than those of mice of the same age inoculated with TOS-V virus.

Discussion

Toscana virus was first isolated from *Phlebotomus perniciosus* collected in Monte Argentario in the Toscana region (Verani *et al.*, 1980). Based on the pathogenicity for laboratory animals, TOS virus was considered distinct from SFN virus already in the original observations, even though these two viruses were closely serologically related. The biological differences described in this study further add to this distinction. In particular it was shown that TOS virus was cytopathogenic for several of the commonly available vertebrate cell culture systems, in which it has been readily propagated, whereas under the same conditions SFN virus produced only slight cytopathogenic changes. A further pronounced biological difference between these two viruses is represented by a different plaque size enhancing effect of DMSO when added to the overlay.

Different response to the effect of DMSO on plaque formation have been reported among the members of Phlebotomus fever group, e.g. DMSO did not enhance plaque formation by those viruses which produced very large plaques (3—5 mm) as compared to those formed by SFN and SFS viruses (0.5—1.5 mm) (McCown *et al.*, 1979). Although there are few published data on biological characteristics of members of the Phlebotomus fever group, thermal and pH lability, and sensitivity to the action of lipid solvents, as described for TOS virus, are consistent with those reported for other *Phleboviruses* (Calisher *et al.*, 1977).

Besides biological differences, SFN and TOS viruses have been registered in the Arbovirus Catalogue as distinct agents on the basis of serological results. Among the *Phleboviruses*, TOS, SFN, and a virus (Tehran virus) recovered from *Phlebotomus papatasi* collected in Iran in 1959 are very closely related, as reported by Tesh *et al.* (1982). They were barely distinguishable by CF test, but were distinct by PRN technique. Antigenic differences between SFN and TOS viruses were also confirmed by serological surveys performed with human sera (Nicoletti *et al.*, 1980). The serological distinctness between TOS and SFN viruses was further demonstrated by a short-incubation neutralization test. The degree of cross-reactivity between SFN and TOS viruses observed by the IFA test is in agreement with data reported for other *Phleboviruses* (Tesh *et al.*, 1982). It follows that this relatively rapid technique is not useful for identifying both new field isolates and specific antibodies in human and animal sera.

The aim of this study was also to ascertain the influence of the host system used for virus isolation on some biological properties of TOS virus. The variability in behaviour of different virus strains in the course of passages in a host is well known (Málková and Narender Reddy, 1975). It has been explained by various degrees of heterogeneity of uncloned virus populations which came into contact with a new environment, e.g. system used for isolation. We have compared the plaque size, kinetics of replication, presence

of auto-interfering activity, efficiency of replication at different temperatures, and virulence for mice of two strains of Toscana virus obtained from the same sandfly pool and freshly isolated and passaged in two different systems, namely in mouse brain and Vero cells. The results showed that after 4—6 passages in a different host system, changes in some of these biological properties can occur. Changes in plaque size, in the amount of auto-interfering activity and of virulence for the white mouse seemed to be the most sensitive indicators of the changes in the environment.

The plaque size in Vero cells seemed to correlate with the virulence for white mice as TOS-MB strain gave small plaques and was more virulent than TOS-V strain which, instead, gave large plaques. This is in accord with experiences with some other viruses (Kimura and Ueba, 1978).

Although assay systems in both suckling mice and Vero cells appeared to be of the same sensitivity (no difference in LD_{50} or PFU titres was observed for the same stock), TOS-MB strain reached regularly higher infectious titres then TOS-V strain. However, we observed a multiplicity-dependent interference phenomenon after infection of Vero cells with TOS-MB but not with TOS-V strain. The interference could be detected by reduction of infectious yield of standard TOS virus and was thus compatible with the biological behaviour of defective interfering particles. We are now further studying the evolution of the interference phenomenon after repeated passages of both strains in vivo and in vitro.

Acknowledgments. The authors are grateful to Dr. M. Grešíková and to Dr. G. B. Rossi for reading the manuscript. This work was supported in part by the grant from Consiglio Nazionale delle Ricerche, Progetto Finalizzato „Controllo delle Malattie da Infezioni" (N. 82.02422.52).

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