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The Latency and Reactivation of Temperature- Sensitive Mutants of Mouse Cytomegalovirus in Different Organs of Mice.

Hazem Akel*, Farouk Al-Quadan, Moh'd Al-Huniti, Suzan Al-Oreibi, Moh'd Ghalib and Sheerin Issa.

Faculty of Allied Health Sciences; Hashemite University.

Abstract

Nine temperature sensitive (*ts*) mutants of mouse cytomegalovirus (MCMV) were compared with wild type (*wt*) for their ability to become latent and then be reactivated in different organs of Swiss mice. Three mutants (*tsm20*, *tsm23* and *tsm27*) failed to replicate in mice and were avirulent. Three other mutants (*tsm12*, *tsm21* and *tsm25*) were of similar virulence to the parental (*wt*) virus. All infected mice with *wt* died without prior infection after seven days, but the mice infected with *ts* mutants were still alive. Titer of virus in hearts, lungs and salivary glands homogenates from mice infected with *tsm15* continued to rise with time. After immunosuppressive treatment, infected mice with *tsm15* and *tsm25* had detectable virus in all tested organs; infected mice with *tsm29* had undetectable virus in spleens and kidneys; whereas, *tsm12* and *tsm21* had detectable virus in lungs and salivary glands. Mutant *tsm10* was unable to be reactivated. Virus recovered from salivary glands of mice infected with *tsm15*, *tsm25* or *tsm29* remained *ts*. These different mutants should prove useful for examining the viral and host factors involved in latency and reactivation.

الملخص

تم مقارنة تسع سلالات حساسة للحرارة من فيروس Cytomegalo مع نظيراتها من الأصل البري لقدرتها على الكمون ثم اعادة النشاط في الأعضاء المختلفة للفئران السويسرية. وقد تبيّن من الدراسة أن ثلاث سلالات(tsm20,tsm 23,tsm27) قد فقدت قدرتها غلى النمو والتكاثر ، وهناك ثلاث سلالات (tsm12,tsm21,tsm25) لها نفس خصائص الأصل البري. كما تبيّن أن جميع الفئران التي حقنت بسلالة الأصل البري قد نفقت خلال سبعة ايام بينما بقيت جميع الفئران التي حقنت بالسلالات الحساسة للحرارة على قيد الحياه. وقد بيّنت الدراسة أن الفيروس الحساس للحرارة tsm 15 يتكاثر مع الوقت أثر حقنه باالرئتين أو الغدد اللعابية أو القلب، كما ظهرمن عدد الفيروسات المتواجدة في مطحون هذه الأعضاء.كما أن حقن الفئران بفيروسات tsm15,tsm 25 بعد علاجها بمثبطات جهاز المناعة اظهر اعدادا من الفيروس يمكن قياسها في جميع الأعضاء التي تم فحصها. كما أن حقن الفئران بفيروس tsm29 تسبب بوجود اعدادا من الفيروس يمكن قياسها في الكلى والطحال. كما ان حقن الفئران بفيروس tsm 12 او tsm21 تسبب بظهور اعدادا من الفيروس يمكن قياسها في الرئتين والغدد اللعابية. كما بينت الدراسة أن الفيروس tsm 10 قد فشل في النمو بعد حقنه. كما أظهرت الدراسةأن الفيروسات tsm 15, tsm25,tsm29 المتحصّل عليها من الغدد اللعابية للفئران قد بقيت ذات طبيعة حساسة للحرارة. كما تظهر هذه الدراسه جدوى دراسة هذه الأنواع من الفيروسات .

Keywords: Cytomegalovirus; mouse; temperature sensitive; latency; reactivation.

1. Introduction

The establishment and maintenance of cytomegalovirus (CMV) latency in infected hosts has been subject of intense investigation for many decades (Tsutsui *et al.*, 2002). However, the mechanisms involved in the induction and maintenance of latency are not fully understood. Virus latency is achieved *in vivo* when infectious viral particles cannot be isolated from the host. *In vitro* induction of CMV latency has been accomplished through the use of viral inhibitors and temperature manipulation (Hummel *et al.*, 2001; Hummel and Abecassis, 2002).

Cytomegalovirus is a β-herpesvirus that is fairly ubiquitous in the human population and causes mild or subclinical disease in healthy individuals (Mocarski, 1996). Human cytomegalovirus (HCMV) has been shown to establish latency in cells of the monocyte/macrophage lineage including hematopoietic progenitor cells (Söderberg-Nauclèr *et al.*, 2001); and there is evidence of persistent HCMV infection in aortic endothelial cells (Fish *et al.*, 1995). The ability of CMV to reactivate from a latent state that is subsequently accompanied by asymptomatic viral shedding can periodically occur in healthy, seropositive individuals; however, the specific cell types from which recurrent virus comes are unknown. A significant amount of CMV morbidity can be attributed to reactivation events that are normally controlled by the immune system, based on the observation that immunosuppressed individuals often suffer from HCMV disease (Kercher and Mitchell, 2002).

Murine cytomegalovirus (CMV) has been used successfully to study parameters of latency in visceral organs such as salivary gland, lung and spleen (Balthesen *et al.*, 1994; Shinmura *et al.*, 1997; Pollock *et al.*, 1995).

* Corresponding author. e-mail :hazemm@hu.edu.jo.

MCMV is similar to HCMV with respect to pathogenesis and the ability to establish or reactivated from latent infections.

Temperature-sensitive (*ts*) mutants, the first generation of conditional mutants in virology, have been helpful and often superior to null mutants in mapping and identifying viral functions (Roizman and Sears, 1996). Random procedures for the generation of *ts* mutants have been described for alphaherpesviruses (Schaffer *et al*., 1970). However, this method did not lead to major findings in betaherpesviruses, as their most prominent member human cytomegalovirus (HCMV), an important human pathogen. Propagation of conditional betaherpesvirus mutants by selection of *ts* alleles is technically difficult, and a specific gene has not been assigned for any of the reported *ts* mutants (Akel and Sweet, 1993). However, one *ts* allele was generated recently for the UL122 gene of HCMV by rational mutagenesis (Heider *et al.*, 2002).

Recently, Sweet *et al.* (1989 and 1993) had described the isolation and preliminary characterization of 25 *ts* mutants of MCMV derived by mutagenesis of virulent wild-type(*wt*) virus with N-methyl-N-nitro-N-nitrosoguanidine. These mutants varied in virulence from a virulent through 10-100 fold less virulent than *wt* virus (Akel and Sweet, 1993; Sandford and Burn, 1988).

Nine mutants out of 25 *ts* mutants were used to study the latency and reactivation of MCMV in various organs of mice.

2. Matrials And Methods

2.1. Mice

Out bred Swiss mice were obtained from Animal House of the University of Sciences and Technology, Irbid, Jordan. Foetuses of sixteen-day old pregnant mice were used to prepare mouse embryo fibroblast (MEF) cultures and for litters to be used for mouse passaged virus. Litters of Swiss mice were used to determine the lethality of mutants of MCMV. Infected and control mice were housed separately.

2.2. Virus

Wild type and mutant viruses were originally supplied by Dr Clive Sweet (Department of Bioscience, Birmingham University, Birmingham, United Kingdom) who had developed them from the Smith strain after many passages *in vivo* in mouse salivary glands (Mims and Gould, 1979). These viruses were cloned in mouse embryo fibroblast cells using terminal dilution as described by Sammons and Sweet (1989). Cloned stocks containing 1.4-3.2 x 10^5 pfu/ml was used for subsequent working.

2.3. Mouse Passaged Stocks of Virus

Groups of twenty of one-week-old Swiss mice were inoculated intraperitoneally (i.p.) with 50 µl of the seed stock of the virus, either undiluted or diluted up to 10 fold in growth medium. Twenty to 26 days later, mice were killed by anaesthetic overdose (Pentobarbiton sodium B. P.) and the salivary glands removed aseptically. These were homogenized in a small volume of growth medium using ultra-turrax T_8 IKA Labortechnik mixer (Germany) to give 10% suspensions, clarified by centrifugation, sonicated, filtered and stored at -70°C. These stocks were labeled mouse passage one (MP1). The procedure was repeated to produce mouse passage two (MP2) stocks using MP1-stock as inoculum.

Growth Curve of Ts Mutants at **37°C-**

Confluent monolayer containing 10^6 cells/well in 24-well multidishes were inoculated with 150 µl of the *ts* mutants in growth medium at multiplicity of infection (moi) of 1. After 60 min adsorption at 37°C cultures were washed with 0.5 ml of phosphate buffered saline (PBS) and overlaid with 0.5 ml of maintenance medium. The multidishes were incubated in a humid atmosphere of 5% CO_2/95% air, in CO_2 incubator at 37°C. At various times (0 hr, 1 hr, 4 hr, 10 hr, 24 hr, 48 hr, 96 hr and 120 hr) after infection, the viruses were harvested and stored at -70°C until yields were assayed at 37°C.

2.4. Determination of Lethality of Ts Mutants for Mice (LD_{50}):

The susceptibility of one-week old Swiss mice to infection with *ts* mutants virus determined by i.p. inoculation of groups of mice with 50 µl of serial 2 fold dilutions of virus in growth medium; groups of 20 mice were used per dilution. Initially the end-point was death of the animal allowing calculation of the 50% lethal dose (LD_{50}) by the method of Reed and Muench (1938). Later the feasibility of non-lethal end points was explored and this is described in the results.

2.5. Measurement of Ts Mutants Titers in Various Organs:

Twenty litters of Swiss mice were harvested on each of the day 2, 4, 7 and 11 post-infection (p.i.) with 300 pfu MCVM. The 300 pfu MCMV was lethal for 95% of Swiss mice, which died at ≤7 days p.i. . Spleens, livers, hearts, lungs, brains and salivary glands were removed aseptically, pooled from each litter and weighed. Thus, each litter of mice gave rise to one sample of each organ. Organs were homogenized and the levels of infectious MCMV were determined by a plaque assay in MEFs as described previously (Sammons and Sweet, 1989), modified to use 24-well trays seeded with 10^6 cells/ well. The results are expressed as the pfu of MCMV per g of tissue.

2.6. Activation of Latent Virus from Various Organs:

Four week-old Swiss mice were inoculated i.p. with 10^2 pfu of MCMV. Control mice were given normal rabbit serum and saline. Infected and control mice kept for one year. Infected mice then were given rabbit antilymphocyte serum (MA Biorpoducts) at a dosage of 0.3 ml twice week and cortisone acetate at a dosage of 125 mg/kg of body weight up to 21 days. Both agents were administered i.p. . At the end of three weeks, the mice were killed, and spleens, livers, hearts, lungs, brains and salivary glands were removed aseptically. Organs were homogenized and the levels of infectious MCMV were determined by a plaque assay in MEM, modified to use 24-weel trays seeded with 10^6 cells/well. The results are expressed as number of positive/ number of tested mice.

3. RESULTS

3.1. Titration of Stocks and Virulence of Mutants

The pfu/LD_{50} of the parental and various mutant viruses grown as stocks by passage in mouse (mouse passage 1 and 2) are shown in Table-1.

Table 1: Viral titration of tissue culture grown, mouse passage 1 and mouse passage 2 stocks, and virulence of *wt* and ts mutants, grown and assayed at 37°C.

issue culture grown stocks				Mouse grown stocks							
				Mouse passage 1			Mouse passage 2				
Virus titer (log_{10} pfu/ml)[a] after assaying at:				Virus titer (log_{10} pfu/ml)[b] after assaying at:			Virus titer (log_{10} pfu/ml)[c] after assaying at:			Virulence (pfu/LD_{50})	
	33°C	37°C	41°C	33°C	37°C	41°C	33°C	37°C	41°C		
wt	4.0	4.2	2.9	6.1	6.1	5.8	6.3	6.1	6.1	≥39	
tsm10	4.2	3.8	<1	2.1	1.0	<1	4.3	3.1	<1	≥205,000	
tsm12	5.0	5.1	1.4	4.3	4.1	<1	5.2	4.7	3.0	≥50,000	
tsm15	5.1	5.0	<1	2.1	3.7	<1	3.2	2.1	<1	≥125	
tsm20	4.2	3.4	1.5	1.1	<1	<1	<1	<1	<1	-	
tsm21	4.0	3.0	<1	3.2	<1	<1	4.2	3.1	1.5	≥12,562	
tsm23	4.2	3.2	<1	1.2	<1	<1	<1	<1	<1	-	
tsm25	4.1	4.6	2.2	3.3	3.0	<1	4.0	3.3	2.0	≥150	
tsm27	4.9	3.3	<1	2.3	<1	<1	3.6	<1	<1	-	
tsm29	4.0	3.3	1.2	2.8	<1	<1	3.5	2.6	<1	≥575	

[a, b and c] Results are expressed as the mean of three samples ± SEM.

Mouse stocks were prepared by i.p. inoculation of one-week-old Swiss mice with non-passaged (to produce mouse passage 1) or mouse-passaged (to produce mouse passage 2) stocks. The LD_{50} of *wt* virus for 2-week-old Swiss mice was 39 pfu in agreement with previously published data (Akel and Sweet, 1993). Three mutants (*tsm20, tsm23* and *tsm27*) were avirulent; their attempt to produce mouse-passaged virus were unsuccessful Table 1. Mutant (*tsm21*) did not replicate when inoculated into mice to produce mouse passage number 1; the other four mutants (*tsm10, tsm12, tsm21* and *tsm25*) showed ability to grow in mice.

Mutants that could be grown in mice showed considerable differences in virulence. Two mutants (*tsm15* and *tsm25*) were 86 and 111 fold less virulent than *wt* virus; one mutant (*tsm29*) was 536 fold less virulent and the remaining mutants (*tsm10, tsm12* and *tsm21*) were 2261 up to ~499961 less virulent than parental *wt* virus.

3.2. Growth curves of mutants

The growth curves at temperature 37°C of wild-type virus and of five mutants (*tsm12*, *tsm15*, *tsm21*, *tsm25* and *tsm29*) each inoculated at multiplicity of 1 are shown in Figure 1. Wild-type virus first produced infectious virus after 50 hrs of onset of infection and reached maximum

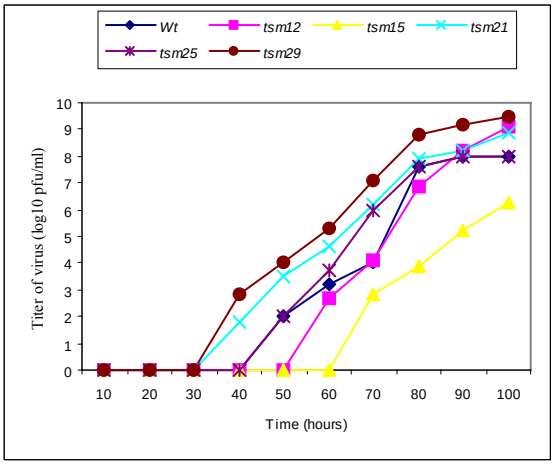

Figure 1. Growth curve of *wt* and ts mutants of MCMV grown and assayed at 37°C. Cells were inoculated at an input multiplicity of 1. Where, *tsm10* was not determined.

virus yields by 90 hrs p.i. Two mutants (*tsm21* and *tsm29*) released infectious virus particles at 40 hrs after infection and virus yields reached maximum yields by 100 hrs. The other mutants (*tsm12*, *tsm15* and *tsm25*) were a little slower and infectious particles did not appear until 60, 70 and 50 hrs i.p. respectively, although virus yields

reached maximum levels by 100 (*tsm12* and *tsm15*) and 90 hrs (*tsm25*) respectively.

3.3. Cumulative Mortality of Wild Type and Mutant Viruses

Table 2 shows the percent cumulative mortality induced by the *wt* virus and *ts* mutants with the lethal dose, 100% deaths generally occurred within 8 days of inoculation as seen with *tsm29*.

Table 2. Cumulative mortality of *wt* and ts mutants of MCMV for one-week old Swiss mice.

Virus	Dose (pfu/0.05 ml)	**Cumulative mortality (%)** Days post infection											
		1	2	3	4	5	6	7	8	9	10	11	12
wt	1,250	0	0	100	100	100	100	100	100	100	100	100	100
	625	0	0	0	70	90	100	100	100	100	100	100	100
	313	0	0	0	40	50	100	100	100	100	100	100	100
	156	0	0	0	0	0	70	90	100	100	100	100	100
	78	0	0	0	0	0	20	90	90	100	100	100	100
	39	0	0	0	0	0	0	0	0	20	52	70	100
	20	0	0	0	0	0	0	0	0	0	0	0	0
tsm10	20,000,000	0	100	100	100	100	100	100	100	100	100	100	100
	10,000,000	0	0	0	0	100	100	100	100	100	100	100	100
	50,000	0	0	0	0	0	0	0	80	80	100	100	100
	205,000	0	0	0	0	0	0	0	0	0	50	70	80
	102,500	0	0	0	0	0	0	0	0	0	0	0	0
tsm12	200,000	0	0	0	0	0	30	50	50	100	100	100	100
	100,000	0	0	0	0	0	0	10	90	100	100	100	100
	50,000	0	0	0	0	0	0	20	42	82	82	82	100
	25,000	0	0	0	0	0	0	0	0	0	0	0	0
tsm15	2,000	0	0	0	50	100	100	100	100	100	100	100	100
	1,000	0	0	0	0	15	100	100	100	100	100	100	100
	500	0	0	0	0	0	20	20	100	100	100	100	100
	250	0	0	0	0	0	0	0	0	80	80	100	100
	125	0	0	0	0	0	0	0	0	0	10	20	30
	63	0	0	0	0	0	0	0	0	0	0	0	0
tsm21	202,000	0	0	40	50	100	100	100	100	100	100	100	100
	101,000	0	0	0	50	80	80	100	100	100	100	100	100
	100,500	0	0	0	0	0	0	60	73	80	80	100	100
	50,125	0	0	0	0	0	0	40	40	40	40	50	70
	25,125	0	0	0	0	0	0	0	0	10	10	40	40
	12,562	0	0	0	0	0	0	0	0	0	5	35	35
	6,281	0	0	0	0	0	0	0	0	0	0	0	0
tsm25	8000	0	0	45	100	100	100	100	100	100	100	100	100
	4000	0	0	30	90	100	100	100	100	100	100	100	100
	2000	0	0	0	0	0	45	60	60	60	100	100	100
	1000	0	0	0	0	0	15	50	60	60	60	100	100
	500	0	0	0	0	0	0	30	43	43	70	80	100
	250	0	0	0	0	0	0	0	20	43	50	50	50
	150	0	0	0	0	0	0	0	0	0	5	35	40
	63	0	0	0	0	0	0	0	0	0	0	0	0
tsm29	5,200	0	0	0	60	100	100	100	100	100	100	100	100
	4,600	0	0	0	30	100	100	100	100	100	100	100	100
	2,300	0	0	0	0	0	60	90	100	100	100	100	100
	1,150	0	0	0	0	0	0	40	40	60	65	65	80
	575	0	0	0	0	0	0	0	30	45	45	45	45
	287	0	0	0	0	0	0	0	0	0	0	0	0

With some viruses, however, the lowest lethal dose took a little longer to kill, e.g. *tsm10, tsm15* and *tsm21* took 10-11 days while *wt* virus, *tsm12* and *tsm25* took 12 days to kill 100% of the animal at the lowest lethal dose.

It appears that there is very sharp dose response curve; generally, an increase in dose of as little as 2 fold can change lethality of the virus from 0% to 100% mortality such as *wt*, *tsm10* and *tsm12*. Even with the other mutants

only 4-6 fold difference in inoculum resulted in a 0-100% difference in mortality.

3.4. Effect of Mutant Infection on Body and Organ Weight

Individual control and infected mice were harvested and then weighed 11 days after inoculation. A minimum of 20 individual weights was used for each group. The mean weight of infected Swiss mice with *wt* was significantly lower than that of control Swiss mice Table 3 .

Table 3. Effect of *wt* and ts mutants of MCMV infection on body and organ weight[a].

	Control	Infected				
		wt	*tsm12*	*tsm15*	*tsm25*	*tsm29*
Mean body weight g±SEM[b]	11.60± 0.9	9.5± 0.44	11.3± 0.22	10.0± 0.1	7.85± 0.24	11.9± 0.2
Mean organ weight mg±SEM Spleen	48± 2	37± 2	45± 8	46± 4	43± 4	45± 3
Liver	250± 0	145± 8	208± 10	240± 10	185± 4	153± 6
Heart	66± 3	50± 4	62± 3	63± 4	55± 4	56± 3
Lungs	121± 5	75± 6	118± 3	122± 1	105± 2	100± 4
Brain	86± 3.0	71± 1	84± 6	84± 4	78± 4	80± 4
Organ weight as a % of mean body weight						
Spleen	0.41	0.39	0.40	0.46	0.55	0.38
Liver	2.16	1.53	1.84	2.40	2.36	1.28
Heart	0.57	0.53	0.55	0.63	0.70	0.47
Lungs	1.04	0.79	1.04	1.22	1.34	0.84
Brain	0.74	0.75	0.74	0.84	0.99	0.67

[a] Mice were inoculated i.p. with 6 pfu of MCMV or diluent alone on the day of 14 after birth and sacrificed for 11 days p.i.

[b] A minimum of 20 individual weights were used for each group.

tsm10 and *tsm21* – not determined.

Infected Swiss mice with *tsm15* and *tsm29* were slightly runted compared to control Swiss mice. The organs harvested from 20 controls and 20 infected suckling Swiss mice, 11 days after inoculation were weighted as a pool for each litter and the mean individual organ weight was calculated. The organ weights in infected mice were either significantly altered or were reduced compared to controls.

3.5. Latency of Mutant Viruses in Various Organs of Mice

Titer of virus in organs of mice infected with *wt* virus and the six *ts* mutants are presented in Table-4. Twenty mice infected with 300 pfu of tissue culture grown of *wt* virus or one of the six *ts* mutants or with no virus. Titer of virus in organs from mice infected with *wt* were at least >1000 fold higher than those for mice infected with *ts* mutants. After one week of infection, all mice infected with *wt* died without prior infection after seven days, whereas mice infected with mutants were still alive but mutants were below detectable level in brain. At seven days after infection, all tested mutants were recovered from spleen and liver. Titer of virus in hearts, lungs and salivary glands homogenates from mice infected with mutant (*tsm15)* continued to rise with time.

3.6. Activation of Mutant Viruses in Various Organs of Mice

To determine if *ts* mutants virus could become activated from a latent state, group of four-week-old mice kept for one year after infection and then subjected to an immunosuppressive therapy. This immunosuppressive regimen activated virus in all tested organs of nearly all twenty mice infected with *wt* virus Table 5, *tsm15* or *tsm25*. Infected mice with *tsm12* and *tsm21*, after immunosuppressive treatment, had detectable virus level in lungs (25% and 20% respectively) and salivary glands (10%). Virus titer in infected mice with *tsm29* subjected to immunosuppressive treatment was very low in spleen (5%), kidney (5%) and Brain (0%). No virus could be detected in almost all organs of any of twenty infected mice with *tsm10* that were subjected to immunosuppression. As seen in Table 6, virus recovered from salivary glands of mice infected with *tsm15*, *tsm25* or *tsm29* remained *ts*.

4. DISCUSSION

The results of our investigation showed various differences between nine mutants compared with *w*t or control (no virus infection): (i) a comparison of *wt* virus and *ts* mutants with form 86 fold up to >499961 fold differences in virulence may help to elucidate virus functions or virion components involved in latency, reactivation and immunosuppression. It remains to be useful in studies of any of the aspects of virulence; (ii) Organ weights of infected mice with *wt* and *ts* mutants were either significantly altered or were reduced compared to control; (iii) Mice inoculated i.p. with either virulent or attenuated MCMV develop latent infection, which we do not understand the latent state and those factors that maintain it or permit reactivation; and (iv) Activation of virus after immunosuppression indicated that all mice

appeared to develop latent infection with either *wt* virus or *ts* mutants of MCMV regardless of the mice strain, the dose of inoculation or the route of inoculation.

Table 4: Kinetics of *wt* and *ts* mutants of MCMV replication in organs after i.p. inoculation of newborn Swiss mice.

Days post* infection, Virus	Titer of virus (log_{10} pfu/gm of tissue±SEM)^					
	Spleen	Liver	Heart	Lung	Brain	Salivary glands
2						
wt	2.38	2.66	2.04	5.75	2	3.65
tsm10	<1	<1	<1	<1	<1	<1
tsm12	<1	<1	<1	<1	<1	<1
tsm15	1.3	1.0	1.95	2.38	<1	2.00
tsm21	<1	<1	<1	<1	<1	<1
tsm25	1.1	1.1	1.0	1.2	<1	1.6
tsm29	<1	<1	<1	<1	<1	<1
4						
wt	4.08	4.43	3.76	3.61	3.39	6.78
tsm10	<1	<1	<1	<1	<1	<1
tsm12	<1	<1	<1	<1	<1	<1
tsm15	1.35	1.6	2.00	2.20	<1	3.53
tsm21	<1	<1	<1	<1	<1	<1
tsm25	1.0	1.1	1.2	2.20	<1	3.20
tsm29	<1	<1	<1	<1	<1	<1
7						
wt	ND	ND	ND	ND	ND	ND
tsm10	<1	<1	<1	<1	<1	<1
tsm12	<1	<1	<1	<1	<1	<1
tsm15	<1	<1	2.15	2.25	<1	3.28
tsm21	<1	<1	<1	<1	<1	<1
tsm25	<1	<1	<1	<1	<1	<1
tsm29	<1	<1	<1	<1	<1	<1
11						
wt	ND	ND	ND	ND	ND	ND
tsm10	<1	<1	<1	<1	<1	<1
tsm12	<1	<1	<1	<1	<1	<1
tsm15	<1	<1	3.0	2.86	<1	3.9
tsm21	<1	<1	<1	<1	<1	<1
tsm25	<1	<1	<1	<1	<1	1.1
tsm29	<1	<1	<1	<1	<1	<1

^ Results are expressed as the mean of the three samples ± SEM.

* Swiss mice were inoculated i.p. with 300 pfu of virus; organs homogenates from twenty mice killed at each times indicated were pooled.

* ND= Not determined.

Table 5. Activation of latent murine cytomegalovirus and dissemination to various organs of mice after immunosuppression with antilymphocyte serum and cortisone.

Virus			Activation[a]						
Strain	Type	Dose (pfu), route of inoculation	Spleen	Liver	Heart	Lung	Brain	Kidney	Salivary gland
wt	Virulent	10^2, i.p.	18/20	19/20	17/20	20/20	20/20	19/20	20/20
tsm10	Attenuated	10^2, i.p.	0/20	0/20	0/20	0/20	0/20	0/20	0/20
tsm12	Attenuated	10^2, i.p.	0/20	0/20	0/20	5/20	0/20	0/20	2/20
tsm15	Attenuated	10^2, i.p.	9/20	11/20	9/20	6/20	2/20	2/20	10/20
tsm21	Attenuated	10^2, i.p.	0/20	0/20	0/20	4/20	0/20	0/20	2/20
tsm25	Attenuated	10^2, i.p.	4/20	5/20	4/20	8/20	2/20	6/20	9/20
tsm29	Attenuated	10^2, i.p.	1/20	3/20	5/20	9/20	0/20	1/20	9/20

[a]Data are no. positive/ no. tested.

The virulence of mouse grown wild-type MCMV for one-week-old mice was ≥39 pfu/LD_{50} which was in agreement with previously published data (Shellam and Flexman, 1986; Sammons and Sweet, 1989; Akel and Sweet, 1993). Three mutants (*tsm20, tsm23* and *tsm27*) were avirulent for mice in that they failed to replicate in salivary glands of these mice. Similar results were obtained with *ts6* described by Sandford and Burns (1988), *ts21* described by Sammons and Sweet (1989) and *tsm5* by Morley *et al*. (2002). Clearly the genus defective in the viruses play a crucial role in their replication *in vivo* and may be involved in cell attachment or entry or more likely in early replication events. This was confirmed by the work of Gill *et al.* (2000) who used reverse transcription polymerase chain reaction (RT-PCR) to detect viral transcripts during latency by using 4 gene markers (IE 1, E 1, gB and GH); Their results indicated that replication of *tsm13* was blocked at a late phase, and *tsm22* was blocked at the immediate early phase. Whereas, *tsm9* and *tsm30* were blocked at a maturation step, probably of capsid formation, as gene transcription of all 4-marker genes occurred at 39°C and 40°C. In contrast, three mutants (*tsm12, tsm21* and *tsm25*) were able to grow in salivary glands of inoculated mice and were attenuated but the level of attenuation could not be determined since the highest dose used did not kill mice.

A major difference between *ts* mutants and parent virus infections was the replication pattern in heart, liver, lung, brain and salivary glands. One-week-old mice were inoculated i.p. with 300 pfu of mouse passaged virus. The *wt* virus was lethal at this dose and animals died within 5-7 days of inoculation; the virus become generalized, infecting hearts, lung, liver, spleen, kidney and salivary glands that was in agreement with previously published data (Furrarah and Sweet, 1994). In contrast, mutant viruses were not lethal at this dose but showed variability in replication in tested organs. For viruses (*tsm10, tsm12, tsm21* and *tsm29*) failed to replicate in any tissue while mutants (*tsm15* and *tsm25*) showed poor viral replication in heart (<3 log10 pfu/gm), lungs (<2.86 log10 pfu/gm) and salivary glands (<3.9 log10 pfu/gm).

Infected mice received an immunosuppressive regimen, after one year of inoculation, known to reactivate latent MCMV. The *wt* virus, as previously showed (Furrarah and Sweet, 1994), was most easily reactivated in that ≥85% of animals exhibited virus from hearts, spleens, livers, kidneys, lungs, brains and salivary glands. Mutants (*tsm12* and *tsm21*) could be reactivated but from fewer animals (lungs [25% and 20%] and salivary glands [10%]). Mutant (*tsm10*) could not be reactivated as an infectious virus after immunosuppression.

Table 6: Temperature sensitivity of the reactivated isolates.

Reactivated virus*	Titer (log10 pfu/ml) of virus at		
	33oC	37oC	40oC
wt	4.8	4.5	3.2
tsm 15	4.9	2.1	<1
tsm 25	3.8	1.2	<1
tsm29	4.0	3.2	1.1

*Salivary gland homogenates from (1) *wt*, nineteen mice; (2) *tsm 15*, eleven mice; (3) *tsm 25*, nine mice; and (4) *tsm29*, nine mice; that reactivated following immunosuppression therapy was pooled for this assay.

While, the mutant (*tsm29*) was able to reactivated but in very low in spleen and kidney (5%). Similar results were seen in six mutants (*tsm1, tsm2, tsm3, tsm4, tsm5* and tsm6) tested by Furrarah and Sweet (1994). All virus isolates from salivary glands of *tsm15, tsm25* and *tsm29* retained the *ts* phenotype. Clearly the *ts* lesion in *tsm15, tsm25* and *tsm29* was not in a gene whose product is important in initiating and maintaining latency.

Identification of all MCMV *ts* mutant genes and their ordering on the genetic map should eventually lead to a complete understanding of the structural and functional organization of the genome. This type of information is essential in order to understand the pathogenicity of the virus.

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