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A MOUSE-ATTENUATED,
TEMPERATURE SENSITIVE MUTANT OF
PICHINDE VIRUS: *IN VIVO* AND *IN*
VITRO CHARACTERIZATION

by

Heidi Gruber

A thesis submitted in partial fulfillment of the
requirements for the degree of

Master of Science

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Immunology

The University of Ottawa

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ABSTRACT

Pichinde virus belongs to the arenavirus family, which harbours several serious human pathogens. This work was performed in order to investigate the basis for the mouse-attenuated phenotype of a temperature sensitive mutant of Pichinde, and to identify potential genetic changes responsible. Since arenaviruses demonstrate a tropism for monocytes and macrophages, the ability of the mutant, *ts13*, to replicate in tissues enriched for these cells was assessed. Compared to the wild type (*wt*) parental virus, the mutant *ts13* displayed markedly reduced replication in resident peritoneal cells (RPC) from infected mice, in both the macrophage-rich adherent population, and the non-adherent population, which is a mixture of monocytes and lymphocytes. This indicated that reduced growth in macrophages may be involved in the reduced growth of *ts13 in vivo*. This was further supported by the observation that the replication of *ts13* was also limited in a macrophage cell line, and in RPC infected *in vitro*. The replication of *ts13* was restricted to an even greater extent in mesenteric lymph nodes (MLN), and in the spleen, which also contains a high proportion of macrophages. Interestingly, both the MLN and spleen are composed largely of lymphocytes, which led to speculation that reduced growth in lymphocytes also played a role in the attenuated phenotype. In continuous murine fibroblast cell lines, *ts13* grew to high titres, and did not display reduced growth relative to the *wt*, a finding which suggested that the restricted growth phenotype was specific for particular cell types. These results were confounded by studies in primary murine embryonic fibroblasts, in which *ts13* replicated to a lesser degree than the *wt*. *Ts13* possesses a single amino acid change in the GP-1 glycoprotein,

relative to the *wt* parent, a property which could influence the efficiency of binding to target cells. During these studies, sequence analyses were pursued further, and two additional non-silent mutations, one in the NP protein, and one in the S segment intergenic region, were discovered. The attenuated phenotype results from the combined effect of all of these mutations. Investigations such as those described here will provide valuable information regarding mechanisms of arenavirus attenuation, and the genetic changes required to limit growth and virulence. Ultimately, this will be of use in the design of effective arenaviral vaccines, which will become imperative in the midst of new and emerging arenaviral pathogens.

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LIST OF ABBREVIATIONS

AdPIC	Pichinde virus strain adapted for virulence in inbred guinea pigs
AHF	Argentine hemorrhagic fever
Asn	asparagine
ATP	adenosine triphosphate
BHF	Bolivian hemorrhagic fever
BHK	baby hamster kidney
CD3, CD4, Thy1.2	T cell surface markers
cDNA	complementary DNA
CNS	central nervous system
conA	concanavalin A
CTL	cytotoxic T lymphocytes
Cys	cysteine
DEPC	diethyl pyrocarbonate
D-MEM	Dulbecco's modified essential medium.
DNA	deoxyribonucleic acid
dNTPs	deoxynucleotide triphosphates
DTT	dithiothreitol
EDTA	ethylene diamine tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
EOP	efficiency of plating
FBS	fetal bovine serum.
GP-1, GP-2	mature arenavirus glycoproteins
GP-C	glycoprotein precursor
HAV	hepatitis A virus
HPIV ₃	Human Parainfluenza Virus Type3
IC	infectious centre

Ig	immunoglobulin
IL-2	interleukin-2
Ile	isoleucine
IP	intraperitoneal
IV	intravenous
kDa	kilodalton
L protein	viral RNA-dependent RNA polymerase
L RNA	large genomic segment
LCMV	lymphocytic choriomeningitis virus
L-gln	L-glutamine.
LPS	lipopolysaccharide
MEM	minimal essential medium.
Met	methionine
MgCl ₂	magnesium chloride
MHC	major histocompatibility complex
MLN	mesenteric lymph nodes.
MOI	multiplicity of infection.
mRNA	messenger RNA
MW	molecular weight
NH ₄ OAc	ammonium acetate
NK	natural killer
NP	viral nucleocapsid protein
ORF	open reading frame
p.i.	post-infection
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
pfu	plaque-forming unit.
Phe	phenylalanine
PMA	phorbol myristate acetate

PMSF	phenylmethyl sulphonyl fluoride
RNA	ribonucleic acid
RNP	ribonucleoprotein
RPC	resident peritoneal cells.
RSV	Respiratory Syncytial Virus
S RNA	small genomic RNA segment
SDS	sodium dodecyl sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
Ser	serine
Thr	threonine
TNF α	tumour necrosis factor alpha
TPB	tryptose-phosphate broth
Tris	Tris(hydroxymethyl)aminomethane
ts	temperature sensitive
VHF	Venezuelan hemorrhagic fever
VSV	vesicular stomatitis virus
vv	recombinant vaccinia virus
VZV	varicella zoster virus
wt	wild type
YFV	Yellow Fever Virus
Z	viral zinc-binding protein

INTRODUCTION

1. ATTENUATION.

1.1 Attenuation Defined.

An attenuated virus is one whose capacity for disease production is virtually eliminated, due either to natural events or to experimental manipulations of the wild type parent (Ellis, 1994). Attenuated viruses are excellent vaccine candidates, if they are also capable of replicating sufficiently to stimulate immune responses similar to those elicited during a natural infection and to prime the host's immune system, conferring protection against the more pathogenic viruses without damaging the host (Bannister and Begg, 1990). Compared to inactivated vaccines, live attenuated vaccines are desirable since they stimulate both humoral and cell-mediated immunity, may require fewer doses, and may provide longer-term protection (Ellis, 1994).

1.2 Production of Attenuated Viruses.

There are several known viruses which are naturally attenuated in humans. One well-documented example is that of vaccinia virus, which is closely related to variola virus, the causative agent of smallpox (Ellis, 1994). This virus produces only mild disease, with minor skin lesions, and replication in the host is sufficient to stimulate the immune system, and thus this virus is the basis for smallpox vaccines (Ellis, 1994). Often viruses which possess non-human animal hosts yet are highly similar to human pathogens are capable of some replication in humans but are naturally attenuated for virulence. Bovine and rhesus monkey rotaviruses, which have been evaluated for use in human vaccines, are examples of such viruses (Redmond et al., 1993).

In most instances, biological manipulations which change the growth phenotype have been required in order to generate attenuated viruses. The passaging of a virus through an unnatural host has been one method used to attenuate virulence (Bannister and Begg, 1990). In adapting to the new host, the virus acquires genetic changes that result in reduced virulence for the original host. This was exemplified by the serial passage of the human pathogen Dengue virus in mouse brain (reviewed in Schlesinger, 1977). An attenuated strain of Yellow Fever Virus (YFV) was also produced, in part, by mouse-passage (Theiler and Smith, 1937).

Classically, serial passage of a pathogenic virus through cells which normally are not encountered by this virus has been employed in the production of attenuated viruses, especially for use as potential vaccines (Jansen et al., 1988; Chanock, 1982). The first virus to be attenuated in this manner was YFV, whose virulence was reduced by extensive passage in primary chick embryo cultures (Hearn et al., 1966). Vaccine strains of measles (Kawana et al., 1970), mumps (Sassani et al., 1991), and rubella (Minekawa et al., 1973) viruses were also cell-culture derived, as were candidate vaccines for Dengue virus (Bhamarapravati et al., 1987) and Hepatitis A virus (Feinstone et al., 1983).

Attenuated viruses have arisen following treatment with chemical mutagens such as 5-fluorouracil, 5-azacytidine, or proflavine (Haspel et al., 1975). Mutations arising from such treatment are random, and those with deleterious effects on virus growth may occur within any protein-coding or non-coding region. Chemical mutagenesis was involved in the generation of a live attenuated candidate vaccine for subgroup A of Respiratory Syncytial Virus (RSV) (Firestone et al., 1996). Like many other chemically-mutagenized viruses, such as strains of influenza virus (Gwaltney et al., 1976), poliovirus (Bellocq et al., 1984), and bunyavirus (Gahmberg, 1984), this attenuated strain is temperature-sensitive.

The technique of cold-adaptation has been utilized with considerable success to derive several attenuated viruses for use as vaccine candidates (Herlocher et al., 1996). This

process involves the adaptation of the virus for replication at suboptimal cold temperatures, such as 25°C, as opposed to physiological temperature (Randolph et al., 1994). Cold-adaptation often results in the accumulation of multiple genetic changes, which contributes to the stability of the attenuated phenotype (Randolph et al., 1994), and many cold-adapted viruses are also temperature-sensitive (Randolph et al., 1994; Snyder et al., 1988). Stepwise cold-passage of influenza virus at progressively lower temperatures has resulted in several attenuated temperature sensitive (*ts*) strains, which are potential vaccine candidates (Maassab et al., 1990). A candidate vaccine strain for Human Parainfluenza Virus Type 3 (HPIV₃) was also developed by cold-adaptation (Ray et al., 1995).

In the case of viruses bearing segmented genomes, attenuated strains have been achieved through genetic reassortment. Reassortant viruses are produced by co-infection of cultured cells with both a virulent parental virus and an avirulent master strain (Griot et al., 1994; Redmond et al., 1993). If reassortant progeny acquire segments containing attenuating mutations, their virulence in the host will be reduced. Genetic reassortants were derived from two California serogroup bunyaviruses, one of which was virulent and the other avirulent (Griot et al., 1993). These were utilized to map segments responsible for reduced neurovirulence and neuroinvasiveness in suckling and adult mouse systems. In the case of influenza virus, a cold-adapted attenuated master strain of influenza A was proposed as a donor of genes for reassortment with virulent wild type viruses for use in vaccine development (Snyder et al., 1988). Ideal influenza reassortants would contain the internal proteins of the avirulent virus, conferring the attenuated phenotype, and the surface glycoproteins of the current wild type virus, which provide full immunogenicity (Ellis, 1994).

In addition to methods which alter the growth phenotype through the use of specific culture conditions, strategies for producing attenuated viruses using recombinant DNA technology are also emerging. These require direct manipulation of the virus genome or cDNA, and often result in substitutions or deletions within regions that are required for the

expression of virulence (Duke et al., 1990). The development of rescue systems, in which mutations can be introduced into viral DNA or cDNA and then incorporated into infectious particles, is expected to contribute greatly to the creation of attenuated viruses (Connors et al., 1995). Mutations may be generated by site-directed mutagenesis, or by removing a portion of DNA using restriction enzymes. Prior to the use of such engineered viruses as human vaccines, it is of utmost importance to identify the mutations which attenuate virulence in humans. This can be accomplished by generating a series of engineered rescued viruses containing all or a subset of the proposed relevant mutations, and then assaying these for virulence in appropriate model systems. In order to be valid vaccine candidates, mutant viruses must be replication competent, such that they can be propagated in cell culture, and can induce adequate immune responses *in vivo*.

1.3 Mechanisms of Virulence Reduction.

Several mechanisms have been proposed to explain how the methods used to attenuate viruses result in reduced virulence. There is considerable evidence linking reduced growth and virulence to temperature-sensitivity, which is defined as the reduced ability or inability of the mutant strain to grow at an elevated, or non-permissive, temperature (Richman and Murphy, 1979). Moreover, the isolation of *ts* mutants has frequently been used to identify attenuated viruses; a *ts* phenotype, in addition to reduced growth in experimental animals, can be considered to be a marker of attenuation (Randolph et al., 1994). *Ts* mutations in influenza viruses, RSV, poliovirus, vesicular stomatitis virus (VSV), vaccinia and measles virus have resulted in decreased virulence (Richman and Murphy, 1979; Haspel et al., 1975). Many such viruses have been exploited in the development of human vaccines. In these cases, attenuated virulence results because the body temperature of the host is non-permissive for growth of the *ts* strain (Richman and Murphy, 1979). The restrictive influence of body temperature has also been demonstrated in instances where both

permissive and non-permissive temperatures exist within the same organism (Richman and Murphy, 1979). For example, the temperature in the upper respiratory tracts of mice or hamsters is 32-34°C, which is permissive, whereas the temperature in the lower respiratory tract is considerably higher, 37°C. *Ts* strains of respiratory viruses, such as influenza A, RSV, and parainfluenza viruses are thus capable of replication in the nasal turbinates of experimental animals, but are unable to replicate in deeper tissues such as the lungs (Snyder et al., 1988; Randolph et al., 1994; Skiadopoulos et al., 1998). This stimulates a localized mucosal immune response, which may not protect against infection, but does prevent the severe respiratory illness associated with replication in the lungs (Randolph et al., 1994). The important contribution of *ts* lesions to the attenuated phenotype is confirmed by the observation that virulence can be restored by the reversion of *ts* mutations to the wild type (Richman and Murphy, 1979). This has been exemplified by the isolation of non-*ts* strains of vaccinia and polio virus from cases of encephalitic disease in human patients originally vaccinated with a *ts* virus (Ehregut et al., 1975; Georgescu et al., 1994).

Additionally, viruses may be attenuated due to more generalized defects in replication. Such mutants often possess changes in essential replication proteins or non-coding regions and thus display suboptimal levels of replication regardless of the cell types examined. In such cases, the restricted replication is not temperature-dependent and is observed even at permissive temperatures. For example, the attenuation phenotype of several *ts* mutants of measles virus in neonatal hamsters was believed to be due to a partial defectiveness of the mutants under permissive conditions, rather than to a restrictive effect of the host body temperature (Haspel et al., 1975).

Although there is frequently a causative relationship between temperature-sensitivity and attenuation, attenuating mutations may be independent of the *ts* phenotype, and may be present in both *ts* and non-*ts* attenuated viruses (Richman and Murphy, 1979). Viruses may be attenuated due to host-range restrictions, in which the virus has a reduced ability to

replicate within the original host species, or has acquired a specificity for a different host. The adaptive processes imposed by passaging the virus extensively, either in an atypical host or in cell culture, would give rise to such host-range restrictions. Examples of attenuated viruses where passage in this manner has increased the virulence for one species, yet reduced the virulence for humans include mumps virus (Cochi et al., 1994), and varicella zoster virus (VZV) (Takahashi and Gershon, 1994).

Oftentimes, the host specificity is maintained, while the specificity for certain cell types is altered. Each virus displays a particular tropism, or specificity for infecting particular cell or tissue types (Tyler and Fields, 1996). Tropism is determined at multiple levels of the virus-host interaction, such as attachment to host cells, and subsequent viral gene expression and replication (Tyler and Fields, 1996). By infecting specific target cells in the host, a virus is capable of initiating a productive infection at the site of primary inoculation or entry; this facilitates dissemination of the virus to other tissues, which also harbour specific target cells (Tyler and Fields, 1996). The growth of the virus in the host may become attenuated if the ability to infect and replicate within these essential cell types is reduced or lost. Such altered tropism may arise from an inability to adsorb to and enter specific cell types or to effectively utilize the replicative or protein synthesis machinery of these cells. Since the growth of the virus would be impaired, the pathological changes, tissue damage and illness normally resulting from infection by the wild type (*wt*) virus would also be limited. Myxoma virus, a poxvirus which induces a lethal myxomatosis disease in European rabbits, displays an early tropism for CD4⁺ T cells. An attenuated mutant myxoma virus strain which was deficient in a protein thought to play a role in host cell specificity and in the productive infection of CD4⁺ T cells has been described (Mossman et al., 1996). This virus produced only an abortive infection in CD4⁺ T cells, but infection did not spread past the site of primary replication, and no severe, degenerative epidermal lesions appeared. Altered tropism has also been proposed for an attenuated mutant of influenza virus whose lethality for chickens was markedly

reduced (Philpott et al., 1989). Although this mutant was detectable in the spleen, it apparently spared cell populations, such as lymphocytes, which were involved in the pathogenesis of the *wt* virus.

1.4 Genetic Basis of the Attenuated Phenotype.

Changes within the viral genome are ultimately responsible for the attenuated phenotype. The participation of all viral genes is usually required for productive growth; this enables the potential for virulence to be fully expressed (Richman and Murphy, 1979). Thus, the functional loss of any gene, particularly one which is essential for host cell entry, genome replication, or morphogenesis of progeny virions, could lead to an attenuation of virulence. The nature and location of attenuating mutations influence whether the reduced virulence results from temperature-sensitivity, host restriction, altered cell tropism, or generalized replicative defects. Attenuation often arises from the combined effects of multiple genetic lesions, each of which can be either *ts* or non-*ts*. The presence of multiple mutations is desirable for live vaccines, since it enhances the stability of the attenuated phenotype and thereby improves the safety of the vaccine (Snyder et al., 1988).

Viral surface proteins, such as the glycoprotein spikes of enveloped viruses, are essential for early interactions with the host cell, and for establishing a productive infection. Since surface proteins mediate such crucial events as binding to cellular receptors, fusion, and cell entry, amino acid mutations which reduce the structural or functional integrity of these proteins have resulted in attenuated viruses (Scolaro et al., 1990). Single amino acid changes in the envelope glycoproteins were sufficient to alter the virulence of Sindbis virus (Davis et al., 1986), rabies virus (Dietzhold et al., 1983), and rubella virus, where mutations resulted in the loss of a potential glycosylation site on the major envelope protein (Nakhasi et al., 1989). The latter attenuated strain demonstrated reduced binding to, and slow entry into, target cells. Two amino acid changes which may impact on protein tertiary structure have

been identified in the fusion (F) glycoprotein of several cold-passaged attenuated strains of RSV, one of which is a vaccine candidate (Firestone et al., 1996). Glycoprotein mutations which result in altered cell tropism have also been observed to cause attenuation, as was shown for the California serogroup bunyaviruses (Griot et al., 1993). Certain reassortant viruses, where the genomic segment encoding the glycoproteins G1 and G2 was derived from the avirulent parental virus, were unable to replicate in the striated muscle of subcutaneously-inoculated mice, and were non-neuroinvasive due to a lack of spread from the site of inoculation. However, when inoculated intracranially, such clones replicated in brain tissue as efficiently as the virulent strains, suggesting that their growth was specifically reduced in muscle cells, and that this altered tropism was the mechanism of attenuation in the subcutaneously-inoculated mice. Amino acid changes proposed to play a role in attenuation have also been identified in the membrane protein of YFV vaccine strains (Wang et al., 1995) and in both the fusion (F) and hemagglutinin-neuraminidase (HN) glycoproteins of HPIV₃ candidate vaccines (Stokes et al., 1993).

Replication proteins, namely DNA and RNA polymerases, play an integral role in the propagation of viral genomes and in the production of progeny virions (Tyler and Fields, 1996). In addition, polymerases may interact with host cell factors to facilitate viral adaptation to and replication within a particular cell type (Matloubian et al., 1993). Several internal virion proteins played a role in the attenuated phenotype of the cold adapted *ts* influenza A strain discussed above, particularly the 3 proteins, PB1, PB2, and PA, thought to be involved in viral RNA replication (Herlocher et al., 1996). The attenuated phenotype was strongly associated with temperature sensitivity, a property which has also been mapped to PB1 and PB2 (Snyder et al., 1988). Several mutations were identified in the RNA polymerase (L) gene of the candidate RSV subgroup A vaccine strain (Firestone et al., 1996), and of cold-passaged, *ts* candidate HPIV₃ vaccines (Skiadopoulos et al., 1998; Stokes et al., 1993), relative to the *wt* parental viruses. In combination with the aforementioned mutations in the

glycoproteins, the polymerase mutations were suggested to contribute to the attenuated phenotype (Stokes et al., 1993; Firestone et al., 1996). Certain of the California bunyavirus reassortants described above possessed the polymerase-encoding segment of the avirulent parent (Griot et al., 1993). These clones were avirulent for adult mice and showed a host-range restriction, as evidenced by their inability to replicate in brain tissue. Finally, the Sabin 1 vaccine strain of polio virus contained at least one mutation within the 3D^{pol}, or RNA-dependent RNA polymerase, region which contributed to the attenuated phenotype (McGoldrick et al., 1995; Georgescu et al., 1995). One particular mutation was located within a short peptide region important for polymerase activity, and was also implicated in the *ts* phenotype (Georgescu et al., 1995).

Other viral proteins, particularly internal proteins, may contain lesions that contribute to reduced virulence, though none of these proteins has been involved as consistently as have surface proteins and polymerases. The relevance of amino acid changes in internal proteins likely depends on the importance of the protein for a productive viral infection. Amino acid mutations in non-structural (NS) proteins of attenuated strains of Rift Valley Fever virus and influenza virus have been shown to contribute to the attenuated phenotype (Vialat et al., 1997; Herlocher et al., 1996). Two studies with a cold-adapted, *ts* attenuated strain of influenza virus have implicated the matrix protein, which may play an important role in the uncoating of the virus following entry into cells, in the attenuated phenotype of this virus (Herlocher et al., 1996; Snyder et al., 1988). Finally, an amino acid change was identified in the nucleoprotein (N) of the RSV candidate vaccine strain discussed above, although the significance of this mutation is not known (Firestone et al., 1996).

Although many attenuating mutations have been localized to protein-coding genes, non-coding regions may also harbour such mutations. This has been exemplified by several members of the picornavirus family. The 5' non-coding region (5'-NC) of these viruses forms an extensive secondary structure, which interacts with host proteins and plays an

important role in genome replication and in cap-independent initiation of translation (Minor, 1992). Mutations within the 5'-NC may therefore have dramatic effects on RNA secondary structure and on viral replication. The major attenuating mutations in all 3 serotypes of Sabin vaccine are located within the 5'-NC (McGoldrick et al., 1995; Guillot et al., 1994), and mutations in the 5'-NC are also present in attenuated strains of hepatitis A virus (HAV) (Taylor et al., 1993), and Mengovirus (Palmenberg and Osorio, 1994). Polio virus also possesses a short 3' non-coding region (3'-NC), which is involved in viral replication (Georgescu et al., 1995). At least one mutation within the 3'-NC has been proposed to contribute to the attenuated phenotype of the Sabin type 1 vaccine (Georgescu et al., 1995). Virulent polioviruses isolated from cases of post-vaccination disease have shown reversion at sites in the 3D^{pol} and 3'-NC (Georgescu et al., 1995).

2. ARENAVIRUSES.

2.1 Classification and Geographical Distribution.

The family *Arenaviridae* contains enveloped, single-stranded RNA viruses which have provided diverse areas for study, from structure and biochemistry to aspects of human hemorrhagic disease. The prototypical member of this family is Lymphocytic Choriomeningitis virus (LCMV) (Lozano et al., 1997). This highly-characterized virus has illustrated such fundamental concepts as MHC-restricted recognition of infected cells by T lymphocytes (Zinkernagel and Doherty, 1974), T cell-mediated immunopathology (Battegay et al., 1992), virus-induced immune suppression (Ahmed et al., 1987), and viral persistence (Evans et al., 1994; Oldstone and Rall, 1993). On the basis of geographical site of isolation, morphology, complement fixation, antigenic relationships, patterns of cross-neutralization, and more recently, RNA sequence data, the arenaviruses have been sub-divided into two groups (Murphy et al., 1969; Wulff et al., 1978; Ruo et al., 1991; Rowe et al., 1970b; Lozano

et al., 1997). The Old World arenaviruses are endemic throughout Africa, and have been isolated from many West-, Central-, and South-African countries (Ruo et al., 1991). Members of this group include the prototype arenavirus lymphocytic choriomeningitis virus (LCMV), Lassa virus, and others (Lozano et al., 1997), as listed in Table 1. Although classified as a member of the Old World complex, LCMV was introduced into the Americas from Africa, and is thus the only arenavirus with a world-wide distribution (Lozano et al., 1997). The New World, or Tacaribe Complex, arenaviruses are native to South America. In addition to Pichinde virus, this group also includes the pathogenic viruses Junin, Machupo, Guanarito, and Sabia, and several viruses which are not associated with human disease, as summarized in Table 1 (Lozano et al., 1997). Sero-surveys of rodents in various regions of South America and the Southwestern United States have facilitated the identification of at least 3 additional New World viruses in the past two years (Bowen et al., 1996a; Fulhorst et al., 1997), Oliveros virus isolated in Argentina, Pirital virus in Venezuela, and Whitewater Arroyo virus in New Mexico (Bowen et al., 1996a; Fulhorst et al., 1997; Fulhorst et al., 1996). Although not the first arenavirus to be discovered in North America, Whitewater Arroyo virus was the first of its kind to be isolated in the Southwestern United States (Fulhorst et al., 1996). A fourth novel New World virus, Pampa virus, has been identified in Argentina, although its identity as a distinct species is questionable, due to its very high sequence homology to Oliveros virus (Lozano et al., 1997). A complete list of all known arenaviruses, and their geographical distribution, is presented in Table 1. Amplification by the polymerase chain reaction (PCR), using primers specific for conserved regions of the arenavirus genome, has enabled the partial sequencing of every known New World arenavirus (Bowen et al., 1996b). This has permitted a detailed analysis of the genetic relatedness among arenaviruses, and has allowed the New World group to be subdivided into 3 phylogenetic lineages, A, B, and C (Bowen et al., 1996b), as summarized in Table 1. These subdivisions were confirmed by two-way serological analysis (Fulhorst et al., 1996).

Table 1. The Reservoir Hosts and Geographical Distribution of the Arenaviruses.

Virus	Reservoir Host	Lineage	Geographical Distribution	Original Reference
OLD WORLD				
LCMV	<i>Mus Musculus</i>		Americas, Europe, Africa	Armsrong et al., 1933
Lassa	<i>Mastomys natalensis</i>		West Africa	Speir et al., 1970
Mopeia	<i>Mastomys natalensis</i>		South-East Africa	Johnson et al., 1981
Mobala	<i>Praomys jacksoni</i>		Central African Republic	Georges et al., 1985
Ippy	<i>Praomys sp.</i>		Central African Republic	Swanepoel et al., 1985
NEW WORLD				
Pichinde	<i>Oryzomys albigularis</i>	A*	Colombia	Trapido and Sanmartin, 1971
Parana	<i>Oryzomys buccinatus</i>	A	Paraguay	Staneck et al., 1972
Flexal	<i>Oryzomys sp.</i>	A	Brazil	Bowen et al., 1996b
Tamiami	<i>Sigmodon hispidus</i>	A	Florida	Jennings et al., 1970
Whitewater Arroyo	<i>Neotoma albigula</i>	A	New Mexico	Fulhorst et al., 1996
Pirital	<i>Sigmodon alstoni</i>	A	Venezuela	Fulhorst et al., 1997
Junin	<i>Calomys musculus</i>	B ⁺	Argentina	Coto, 1974
Machupo	<i>Calomys callosus</i>	B	Bolivia	Johnson et al., 1966
Guanarito	<i>Zygodontomys brevicauda</i>	B	Venezuela	Salas et al., 1991
Tacaribe	<i>Artibeus sp.</i>	B	Trinidad	Downs et al., 1963
Amapari	<i>Oryzomys gaeldi, Neacomys guianae</i>	B	Brazil	Pinheiro et al., 1966
Sabia	<i>Unknown</i>	B	Brazil	Coimbra et al., 1994
Latino	<i>Calomys callosus</i>	C [#]	Bolivia	Webb et al., 1975
Oliveros	<i>Bolomys obscurus</i>	C	Argentina	Bowen et al., 1996a
Pampa	<i>Bolomys sp.</i>	?	Argentina	Lozano et al., 1997

* Fulhorst et al., 1997

⁺ Bowen et al., 1996b

[#] Bowen et al., 1996a

Arenaviruses are maintained in nature by persistent infection of their vertebrate hosts (Rawls et al., 1981). Generally, each arenavirus is associated with a relatively narrow geographical region, and exists as a reservoir in a specific rodent species or genus. An exception to this is Tacaribe virus, whose putative reservoir is the *Artibeus* species of bat (Rawls and Leung, 1979). Whereas some viruses share the same host species, others have a unique host. Table 1 lists the known reservoir host for each arenavirus. Intraspecies transmission is believed to occur congenitally or neonatally, and such vertically-infected animals develop a lifelong chronic, persistent infection (Rawls et al., 1981). These infections are asymptomatic, with minimal damage to tissue. Infected animals are characterized by lifelong viremia, few or no detectable neutralizing antibodies, and shedding of infectious virus in secreta and excreta (Rawls et al., 1981).

2.2 Virion Structure.

Viewed by electron microscopy, arenavirus particles are pleomorphic, with a roughly spherical form (Burns and Buchmeier, 1991). The granular appearance of these virions gave rise to the name arenavirus, which developed from *arenosus*, the Latin word for sandy (Rowe et al., 1970a). The virus has an average diameter of 90-120 nm, and is surrounded by a host cell-derived lipid envelope (Carballal et al., 1988). The viral envelope is studded with club-shaped spikes, approximately 5- to 10- nm in length, which are composed of the two glycoproteins, GP-1 and GP-2 (Burns and Buchmeier, 1991). Within each virion are two species of single-stranded viral RNA, designated L and S, whose molecular weights are roughly 2.5×10^6 daltons and 1.1×10^6 daltons, respectively (Ramsingh et al., 1980). Each RNA molecule is associated with 3 species of protein: a nucleocapsid protein (NP), an RNA-dependent RNA polymerase (L), and a zinc-binding protein (Z) (Salvato et al., 1992;

Buchmeier and Parekh, 1987; Young and Howard, 1983). This complex constitutes the viral ribonucleoprotein (RNP) core (Salvato et al., 1992; Buchmeier and Parekh, 1987). Incorporated within virions are various host cell-derived macromolecules, including several classes of ribosomal RNA, with sedimentation coefficients of 28 S, 18S, and 4 - 6 S (Harnish et al., 1981), as well as ribosome-like structures which give rise to the sandy appearance of the virus particles (Young and Howard, 1983). Following is a more complete account of each viral protein.

The glycoproteins are produced by post-translational cleavage of a glycoprotein precursor, GP-C (Buchmeier and Oldstone, 1979). Three prominent hydrophobic domains were identified in the GP-C of LCMV (Buchmeier and Parekh, 1987). One of these is located at the amino terminus of the protein, and is believed to form a signal sequence. Another domain is situated near the carboxy terminus, and is thought to serve as a membrane anchor. GP-C is N-linked glycosylated, but the number of glycosylation sites varies for different viruses (Buchmeier and Parekh, 1987). In the trans-Golgi or post-Golgi complex, GP-C is proteolytically cleaved to yield GP-1 and GP-2 (Wright et al., 1990). The proposed site of cleavage is a pair of basic amino acids (Wilson and Clegg, 1991; Buchmeier et al., 1987). GP-1, MW 44-64 kDa, is contained within the amino-terminal portion of GP-C, whereas the carboxy-terminal portion harbours GP-2, MW 35-41 kDa (Buchmeier et al., 1987). Buchmeier and Oldstone (1979) have shown that the cleavage event is required for the transport of the two mature glycoproteins to the surface of infected cells, and consequently, for their incorporation into virions. However, the detection of uncleaved GP-C in virions, although irregular, has been reported (Buchmeier et al., 1978; van der Zeijst et al., 1983).

The native form of GP-1 is a homotetramer, whose structure is maintained by disulphide bonds between monomers, and also within the monomer itself (Burns and Buchmeier, 1991). GP-1, which is a peripheral membrane protein (Burns and Buchmeier,

1991; Buchmeier and Oldstone, 1979), is believed to mediate the binding of arenaviruses to host cell receptors (Borrow and Oldstone, 1992). Among the arenaviruses, GP-1 is the least conserved protein, and monoclonal antibodies specific for epitopes on GP-1 are far less cross-reactive than those specific for GP-2 or NP (Ruo et al., 1991). It has been demonstrated that epitopes for antibodies which neutralize virus infectivity are present on GP-1 (Parekh and Buchmeier, 1986; Bruns et al., 1983).

GP-2 also forms a homotetramer (Burns and Buchmeier, 1991). GP-2 has been identified as an integral membrane protein, possessing an externally-exposed portion, a membrane-spanning domain, and a carboxy terminus which extends into the virion (Buchmeier and Parekh, 1987). Cross-linking studies have shown that GP-2 forms complexes with the nucleoprotein, NP (Burns and Buchmeier, 1991). These interactions occur on the inside of the virion, and are thought to be mediated by the carboxy-terminus of GP-2. In infected cells, the association of GP-2 with the ribonucleoprotein core may play a role in virion assembly and budding (Burns and Buchmeier, 1991). GP-2 is a more highly conserved protein than GP-1 (Clegg, 1992; Ruo et al., 1991). It is broadly cross-reactive, and contains one antigenic site which is conserved across both New and Old World viruses (Ruo et al., 1991; Weber and Buchmeier, 1988). Although GP-1 is the principle target of neutralizing antibodies, GP-2-specific neutralizing antibodies have been reported in the case of Lassa and Mopeia viruses (Ruo et al., 1991).

NP is a 63 - 68 kDa non-glycosylated protein (Buchmeier and Parekh, 1987). It is the most abundant viral protein, and is localized to the RNP core (Veza et al., 1977; Buchmeier et al., 1978). Individual molecules of NP form nucleosome-like structures, 4 to 5 nm in diameter, along the viral RNA segments (Young and Howard, 1983). Bruns et al. (1986) reported that a fraction of the NP is present in a phosphorylated form, which may play a role in regulating viral transcription and replication. Because of its association with GP-2,

NP may also be involved in virion morphogenesis (Burns and Buchmeier, 1991). Like GP-2, NP is also a highly conserved protein, containing many stretches of amino acids that are preserved among the arenaviruses, and demonstrating considerable immunological cross-reactivity (Albarino et al., 1997).

The L protein, also a component of the RNP core, has been proposed as the viral RNA-dependent RNA polymerase (Lukashevich, 1997; Buchmeier and Parekh, 1987). This protein has an approximate molecular weight of 200 - 250 kDa (Lukashevich et al., 1997; Rossi et al., 1996; Harnish et al., 1981). Lukashevich et al. (1997) have identified 3 conserved amino acid motifs between the L protein of Lassa virus, and those of the other arenaviruses, such as LCMV (Salvato and Shimomaye, 1989), Tacaribe (Iapalucci et al., 1989a), and Pichinde (D.G. Harnish, S. Zheng, and S. Polyak, unpublished results), whose L genes have been sequenced. These motifs are also shared by the RNA-dependent RNA polymerases of other segmented, negative-strand viruses, such as the bunyaviruses (Muller et al., 1994), confirming the identity of the L protein as the viral polymerase.

The last arenaviral protein to be characterized was discovered by RNA sequence analyses, which revealed the existence of a small open-reading frame (ORF) on the L RNA segment, encoding an 11 kDa polypeptide (Iapalucci et al., 1989b; Salvato and Shimomaye, 1989). Designated Z, or p11, this protein has been defined for Tacaribe, LCMV, Pichinde and Lassa (Djavani et al., 1997). Salvato et al., (1992) have shown that p11 is present in 400 copies per virus particle, and that it is a structural protein, which is hydrophobic in nature and associated with the RNP core. *In vitro*, p11 has been shown to bind Zn^{2+} (Salvato and Shimomaye, 1989). Further, the amino acid sequence reveals cysteine-rich motifs, forming a RING finger type of zinc-binding domain (Iapalucci et al., 1989b; Djavani et al., 1997). Such domains play a role in RNA binding, and similar structures have been observed in many eukaryotic transcription factors (Iapalucci et al., 1989b). It has therefore been proposed that

p11 is involved in RNA transcription, and that its function may be a regulatory one. Experiments with Tacaribe virus have demonstrated that p11 was essential for both mRNA and genomic RNA synthesis (Garcin et al., 1993). However, it appeared to play different roles in the two processes. The Z protein of LCMV has been observed to bind to the cellular oncoprotein promyelocytic leukemia (PML), and is capable of re-locating this protein to the cytoplasm, forming large cytoplasmic bodies (Borden et al., 1998). Although the consequences of this interaction are not known, this association may somehow influence the physiological effects of arenavirus infections.

2.3 Genetic Organization and Genome Replication.

The arenavirus genome consists of two single-stranded RNA species, L (large) and S (small) (Garcin and Kolakofsky, 1990). Sequencing studies have revealed that arenaviruses employ an ambisense coding strategy (Wilson and Clegg, 1991; Clegg et al., 1990; Romanowski et al., 1985; Auperin et al., 1984), meaning that viral messages are produced from both the genomic RNA and the viral-complementary anti-genomic RNA, which is a replicative intermediate (Garcin and Kolakofsky, 1990). The S segment encodes both the NP gene and the GP-C gene, in two non-overlapping open reading frames (ORFs) of approximately equal lengths (1500-1700 bases) (Clegg et al., 1990; Auperin et al., 1984). Similarly, the L segment encodes both the L gene and the Z gene (Lukashevich et al., 1997; Djavani et al., 1997). The two non-overlapping ORFs within the L segment, however, are greatly dissimilar in size, with the L protein encoded by 6630-6660 bases and the Z protein by only 270-300 bases (Lukashevich et al., 1997; Iapalucci et al., 1989b; Salvato and Shimomaye, 1989). The NP and L proteins are produced from mRNAs complementary to the genome, and which are transcribed from the 3' ends of genomic RNA (Auperin et al., 1984; Iapalucci et al., 1989a). The GP-C and Z proteins are produced from mRNAs which are virus-sense, and are transcribed

from the 3' ends of anti-genomic RNA (Auperin et al., 1984; Iapalucci et al., 1989b). GP-C is synthesized as a mannose-rich glycoprotein, which is transported through the Golgi complex, and subjected to various stages of oligosaccharide trimming and modification (Buchmeier et al., 1987), prior to cleavage in the the trans-Golgi or post-Golgi complex (Wright et al., 1990). Between the termination codons of the two genes on each segment lies a short intergenic region, rich in C and G nucleotides, which is predicted to form a hairpin structure stabilized by base-pairing between complementary residues (Iapalucci et al., 1989b; Auperin et al., 1984). This region is believed to act as a transcription termination signal, since the 3' ends of the individual mRNA molecules have been mapped to this region (Southern, 1996). In addition, it is thought to be involved in maintaining the balance between mRNA transcription and genome replication.

At the 5' and 3' termini of both viral genomes and anti-genomes are 19 highly conserved nucleotides, which are complementary save for two mismatches and which permit the circularization of RNA segments (Auperin et al., 1982a and 1982b; Salvato and Shimomaye, 1989). This stretch of nucleotides likely contains a polymerase binding site, which is necessary for the initiation of mRNA and full-length RNA synthesis from both genome and anti-genome templates (Southern, 1996).

Following binding of virions to host cells, and probable entry via endocytosis, the viral RNP is released into the cytoplasm (Castilla et al., 1994; Borrow and Oldstone, 1994). Attempts to directly translate genomes using *in vitro* systems have been unsuccessful, indicating that transcription is a preliminary requirement for gene expression (Leung et al., 1977). Transcription of the NP gene from the S segment commences very early in infection, well before the onset of replication (Fuller-Pace and Southern, 1988; Bishop and Auperin, 1987). The NP mRNA is presumably generated by the polymerase molecules present within the RNP, and is subsequently translated to yield the NP protein. Given that the L gene is transcribed in an analogous way from the L

segment, its kinetics may parallel those of NP (Southern, 1996).

Initially, the presence of the strong secondary structure in the intergenic region prevents the synthesis of full-length RNA. It has been suggested that anti-termination is required in order for genomic replication to occur, and that this is dependent upon the accumulation of a viral gene product, possibly NP (Auperin et al., 1984). Replication begins with the synthesis of an anti-genome, which initiates at the genomic 3' terminus and results in the generation of a full-length RNA strand which is complementary to the genome (Garcin et al., 1993). This molecule in turn is the template for the synthesis of full-length genomes. Primer-extension analyses have indicated that the 5' ends of arenavirus genomes and anti-genomes contain a single non-templated nucleotide, which is invariably a tri-phosphorylated G-residue (Garcin and Kolakofsky, 1990). Several models, each of which requires the dinucleotide pppGpC as a primer, have been proposed to explain this (Garcin and Kolakovsky, 1990). These employ concepts such as polymerase slippage and the use of host RNAs as a source of dinucleotide primers.

The synthesis of GPC protein does not occur until replication has begun and anti-genomic RNA strands are available to serve as templates for the transcription of GPC mRNAs (Southern, 1996). This was supported by the demonstration that inhibiting replication through the use of protein synthesis inhibitors also inhibited the production of GPC mRNA (Franze-Fernandez et al., 1987). Thus the ambisense nature of the viral genome allows the synthesis of the NP and GPC proteins, and that of the L and Z proteins, to be independently regulated. This may contribute to the establishment of persistent infections, as evidenced by studies of cells persistently infected with either Tacaribe virus or Pichinde virus. NP was produced in vast quantities, whereas the synthesis of GPC appeared to be specifically inhibited, implying a differential regulation of the two proteins (Southern, 1996; Auperin et al., 1984).

It should be noted that arenaviral mRNAs are capped but not polyadenylated

(Singh et al., 1987; Southern et al., 1987). Primer extension studies have revealed that the 5' termini of these RNA species contain from 3 to 9 non-templated nucleotides (Polyak et al., 1995). Sequencing of individual mRNA clones indicates that these nucleotides are heterogeneous (Polyak et al., 1995; Garcin and Kolakofsky, 1990). A cap-snatching mechanism of transcription initiation has not been discounted.

2.4 Arenavirus Biology and Pathogenicity.

Although the *Arenaviridae* produce asymptomatic infections in their natural rodent hosts, several members are the etiological agents of serious human hemorrhagic diseases (Clegg et al., 1990). Humans become infected by contact with infectious rodent excretions, either through skin abrasions, or by the inhalation of contaminated aerosols (Carballal et al., 1988). To date, the known pathogens are LCMV and Lassa virus among the Old World group, and Junin, Machupo, Guanarito, and the recently-discovered Sabia virus, among the New World group (Lozano et al., 1997; Park et al., 1997).

Infection with LCM virus results in a broad spectrum of clinical manifestations, from mild or inapparent symptoms to systemic febrile illness, aseptic meningitis, encephalomyelitis, and occasionally, fatality (Walker and Murphy, 1987). The prevalence of LCMV-associated disease is uncertain (Park et al., 1997), though LCMV has been associated with cases of viral central nervous system disease in the developed world, particularly in populations in the United States and in Europe (Clegg, 1992). LCMV has also been implicated in laboratory-acquired infections (Clegg, 1992; Oldstone, 1987) and in illnesses acquired from contact with infected pet hamsters (Oldstone, 1987). Lassa virus, which is endemic in West Africa, is considered a very dangerous arenaviral pathogen (Lukashevich et al., 1997). It causes Lassa fever, which is frequently characterized by febrile illness and fatal systemic disease (Walker and Murphy, 1987). Antibody to Lassa has been observed in up to 50% of populations in West Africa, and infections with this virus account for half the hospital admissions in some

communities (Clegg, 1992; Walker and Murphy, 1987). The mortality rate among these hospitalized patients is 15-20% (Walker and Murphy, 1987), but lower in milder cases. Serious consequences are associated with infections acquired during pregnancy; Lassa virus has been identified as the most common cause of septic abortion in African hospitals studied (Price et al., 1986). Among pregnant women, infections developed in the third trimester were associated with a greater incidence of mortality.

The South American arenaviruses Junin, Machupo, and Guanarito produce severe hemorrhagic diseases, with cardiovascular, renal, immunological, and neurological consequences (Lozano et al., 1997). As with the Old World viruses, humans are infected by contact with contaminated rodent excreta, although the occurrence of inter-person transmission has been reported for Lassa and Machupo viruses, and very rarely for Junin virus (Vainrub and Salas, 1994; Oldstone, 1987). Argentine hemorrhagic fever (AHF), whose causative agent is Junin virus, is endemic in an increasingly large agricultural and cattle-raising region in Argentina (Carballal et al., 1988; Vainrub and Salas, 1994). Between 1982 and 1987, Junin virus caused an average of 360 cases of AHF per year (Carballal et al., 1988). Of AHF cases, 10-16% prove fatal, and such subjects show severe CNS involvement (Carballal et al., 1988). Mortality can be reduced to 1-2% with the administration of immune convalescent plasma, however neurological complications arise in a small fraction of cases (Clegg, 1992). Bolivian hemorrhagic fever (BHF) is caused by Machupo virus (Bowen et al., 1996b). Unlike AHF, BHF is predominantly house-associated (Vainrub and Salas, 1994), and is not endemic. A large outbreak of BHF developed in Bolivia between 1960 and 1964, affecting greater than 500 people, and causing mortality in 18% of cases (Vainrub and Salas, 1994). Although a rodent eradication program implemented in the endemic area in 1964 dramatically decreased the incidence of disease, sporadic cases may still occur (Vainrub and Salas, 1994).

In 1989, a new illness, initially suspected to be Dengue hemorrhagic fever, began to appear in rural regions in central Venezuela (Salas et al., 1991). The etiological agent of the disease was later determined to be a newly-described arenavirus, Guanarito, which was isolated from tissue samples from fatal cases (Salas et al., 1991). Referred to as Venezuelan hemorrhagic fever (VHF), this illness resulted in a fatality rate of 34% during an outbreak that occurred between 1989 and 1991, and affected 88 people (Vainrub and Salas, 1994). VHF appears to be acquired near or within houses, as for BHF and Lassa fever (Vainrub and Salas, 1994). A novel pathogen, Sabia virus, was recently isolated in Brazil, from a fatal case of hemorrhagic fever which was first thought to be yellow fever (Coimbra et al., 1994). Sabia virus has since caused two non-fatal, but severe, laboratory-acquired infections (Coimbra et al., 1994; Gonzalez et al., 1996).

Interestingly, all of the known New World pathogens are contained within phylogenetic lineage B, one of the 3 lineages described by workers at the Centres for Disease Control (Bowen et al., 1996b). Also belonging to lineage B is Tacaribe virus, which is not a usual pathogen, but has caused one laboratory-acquired illness. With current methods for deriving RNA sequence information from new isolates, it is possible to apply the above phylogenetic analysis to novel arenaviruses (Bowen et al., 1996b; Lozano et al., 1997). Isolates identified within this lineage would be suspicious, and would be handled with caution. This would prove valuable in the detection of emerging pathogens prior to outbreaks of disease in humans.

The pathogenesis of the virulent arenaviruses is not well understood. As observed in human disease cases, the arenaviruses replicate in lymphoid and hemopoetic tissues (Carballal et al., 1988). Junin virus, for example, replicates in the spleen, lymph nodes, bone marrow, liver, and lung, producing a constant viremia (Vainrub and Salas, 1994; Gonzalez et al., 1980). Monocytes and macrophages were identified as frequent sites of viral involvement in tissues, in AHF and other arenaviral illnesses (Peters et al., 1989; Gonzalez et al., 1980).

Ambrosio et al. (1990) demonstrated that circulating blood monocytes are targets of viral replication, and that these contribute to viral spread during the acute phase of AHF. Infection of monocytes and macrophages is believed to alter the immune antiviral response, and may be involved in immune suppression, as well as in the development of persistent infections (Polyak et al., 1995; Friedlander et al., 1984). Given that arenaviruses generally do not induce cytopathic changes in affected tissues, it has been proposed that soluble mediators, possibly produced by macrophages, whose functions are altered during arenavirus infections, were involved in pathogenesis (Peters et al., 1989). A role for interferon- α in AHF was suggested by the elevated levels of this cytokine in cases of disease (Levis et al., 1984). In Lassa virus-infected humans and animals, considerable endothelial cell injury has been reported, although this is due to biochemical alterations, rather than to overt tissue destruction (Fisher-Hoch et al., 1987). This is believed to contribute to altered homeostasis in the blood vessels, leading to leakage of fluids and electrolytes, and predisposing to hemorrhage.

Antibodies detectable by immunofluorescence or complement fixation are detectable within days of infection (Clegg, 1992). These have been found to react with both the viral nucleoprotein and the glycoproteins. Neutralizing antibodies to the Latin American viruses are established within 4-6 weeks of infection, although months are required for the establishment of even modest levels of such antibodies to Lassa virus (Peters et al., 1987). Convalescence and reduction in viremia often begin before significant quantities of neutralizing antibodies are detected, which suggests that cell-mediated mechanisms are responsible for viral clearance and for recovery from infection (Clegg, 1992). Studies of LCMV infection of mice have confirmed the importance of cytotoxic T lymphocytes (CTLs), since mice which were unable to produce functional CTLs were unable to clear the virus, and became persistently infected (Zinkernagel and Doherty, 1979). Nevertheless, both the humoral and cell-mediated responses appear to be involved in the development of immunity

to arenaviral infections (Peters et al., 1987; Maiztegui et al., 1979).

Few practicable arenavirus vaccines have been described. The use of inactivated virus preparations as immunogens has shown little promise (Clegg, 1992). Inactivated Junin and Machupo viruses were difficult to prepare in sufficient quantities, and inactivated forms of Lassa virus demonstrated poor protection in experimental animals, and in some cases exacerbated disease (reviewed in Peters et al., 1987). Non-pathogenic, yet serologically related viruses have been used effectively to confer cross-protection against the more pathogenic viruses, as proven in animal models. Immunization of guinea pigs and primates with Tacaribe virus produced few signs of illness, yet protected against Junin virus challenge (Coto et al., 1967). Similarly, immunization of guinea pigs or primates with Mopeia or Mobala virus provided sufficient protection against challenge with Lassa virus (Peters et al., 1987; Walker et al., 1982). However, the possibility of persistent infection, along with uncertainties associated with the true pathogenic nature of many of these viruses has curtailed the possibilities for use in human vaccines (Clegg, 1992). The concept of using recombinant vaccinia viruses (vv) expressing individual arenaviral genes as immunogens has also shown considerable vaccine potential. Vv expressing either the NP or GPC of Lassa virus have successfully protected infected guinea pigs against Lassa virus challenge (Morrison et al., 1989). In addition, inbred mouse strains were protected against a lethal challenge with LCMV by prior immunization with vv recombinants expressing the LCMV NP or GPC protein (Hany et al., 1989). Previous studies had demonstrated that CTL recognition sites were present on both of these LCMV proteins (Whitton et al., 1988). This was consistent with the discovery that protection by NP- or GPC- expressing vv was largely CTL-mediated.

The production of vaccines by the process of attenuation has met with limited success. The only known candidate vaccine for an arenavirus is an attenuated strain of Junin virus (Clegg, 1992). This strain is designated Candid 1, and was derived from the virulent XJ strain by passage in certified FRHL-2 cells (Barrera Oro and Eddy, 1982). Proven safe and

immunogenic in vaccinated guinea pigs and rhesus monkeys, and in human volunteers, this potential vaccine is presently undergoing additional trials in the AHF endemic region. The attenuation of Machupo and Lassa viruses for use as vaccines was attempted, although these strains are not in use, due to concerns about reversion to virulence (Peters et al., 1987).

With the discovery of such emerging arenaviral pathogens as Guanarito and Sabia viruses, the public health threat of the arenaviruses is becoming evident. The new arenaviruses Oliveros and Whitewater Arroyo are of unknown pathogenic potential, since they were isolated through rodent survey programs rather than from cases of human illness, (Bowen et al., 1996a; Fulhorst et al., 1996). The most recently-described arenavirus, Pirital, was isolated from the rodent species *Sigmodon alstoni*, within the region endemic for VHF (Fulhorst et al., 1997). It was thus proposed that some cases of VHF may actually have been caused by Pirital virus, and not Guanarito virus. For this reason, it will be important to address the risk of Pirital virus to human health. Considerable evidence suggests that arenaviruses are quite prevalent in North America. Of rodents surveyed in the southern United States, at least 3% demonstrated antibodies which reacted with Tacaribe complex arenaviruses (Kosoy et al., 1996). Antibody-positive rodents were detected in 5 *Neotoma* species; depending on the species, from 7 to 18% of rodents were positive. Although the arenavirus associated with *Neotoma albigula* is thought to be Whitewater Arroyo virus (Fulhorst et al., 1996), the possibility exists that each other *Neotoma* species harbours a novel virus (Kosoy et al., 1996). In light of these emerging viruses, some of which may be potential pathogens, there is a growing concern about human exposure, and the need for effective vaccines is becoming more urgent.

2.5 Attenuation among the Arenaviruses.

To date, few attenuated arenaviruses have been described. A mouse-attenuated variant of the Armstrong strain of LCMV was generated from persistently-infected L-

929 cells (Bruns et al., 1997). This variant failed to produce neurological disease in intracranially inoculated mice, and was capable of preventing the replication of the *wt* virus, thereby protecting mice against a fatal challenge. The elimination of *wt* virus was mediated by accelerated activation of CTLs, as well as by neutralizing antibodies, and an extensive early production of interferon- α .

Attenuated strains of Junin virus have been more widely described. The first attenuated Junin virus was designated XJCl3, and was generated by cloning of the parental *wt* in MA-111 cells, followed by subsequent passage in mouse brain (Guerrero et al., 1969, cited in Contigiani et al., 1993). Using the guinea pig model, it was shown that the attenuated strain exhibited a different cell tropism relative to the *wt* (Laguens et al., 1983). Whereas *wt* virus was detected in the spleen in both the macrophages and dendritic cells, XJCl3 was detected only in dendritic cells. These results demonstrated the importance of macrophages in the establishment of an infection with pathogenic consequences. This strain was also shown to be attenuated in rhesus monkeys, and in human volunteers (Contigiani et al., 1993; Avila et al., 1984). However, since the cells used to propagate this strain were not among those recommended for vaccine production, human trials were terminated (Contigiani et al., 1993). The Candid #1 vaccine strain was subsequently generated by passage of the parental virus in fetal rhesus lung (FRhL-2) cells (Barrera Oro and Eddy, 1982). Demonstrated to be highly attenuated in guinea pigs, rhesus monkeys, and human beings, this candidate vaccine was employed in an extensive field trial in the AHF endemic area (Maiztegui et al., 1990). It was reported by Contigiani et al. (1993) that certain individual clones derived from that Candid #1 strain were either more or less attenuated for mice than the Candid 1 parent. This suggested that the strain was heterogeneous, and was a mixture of different viral subpopulations, emphasizing the importance of careful scrutiny of potential candidates.

Chemical mutagenesis of the XJCl3 strain generated a series of *ts* mutants, most of which were strikingly attenuated for virulence in neonatal mice (Ceriatti et al., 1986). One

attenuated mutant, C167, demonstrated an inability to spread to other tissues from the site of inoculation, the brain (Scolaro et al., 1990; Scolaro et al., 1989). The reduced spread was found to be due to a host-range restriction, as evidenced by the markedly reduced replication in cultured murine cells. This reduced growth appeared to involve binding of C167 to murine cells, and its subsequent internalization.

3. PICHINDE VIRUS.

3.1 Isolation of Pichinde Virus: Relationship to the Other Arenaviruses.

Pichinde virus was first isolated from the rice rat *Orizomys albigularis* in the Pichinde Valley of Colombia (Trapido and Sanmartin, 1971; Mifune et al., 1971). On the basis of its ability to produce a chronic infection in its host, as well as its growth kinetics in cultured cells, its response to inhibitors of viral replication, its sensitivity to lipid solvents, and the size and morphology of the virions, Pichinde was classified as a member of the arenavirus family (Mifune et al., 1971). The complete genetic sequence of Pichinde virus is known (Polyak, S.J., S. Zheng, and D.G. Harnish, unpublished results; Auperin et al., 1984). This permits sequence comparisons with novel arenaviruses, and provides a reference for sequence analyses of Pichinde variants with altered phenotypes.

Through phylogenetic analyses, Pichinde virus was found to be the most ancestral member of the Tacaribe complex, and was grouped within lineage A, with Parana, Flexal, Tamiami, Whitewater Arroyo, and Pirital viruses (Bowen et al., 1996a,b).

In enzyme-linked immunosorbent assays (ELISA), Pichinde antigens were most cross-reactive with antibodies to Parana virus (Fulhorst et al., 1997; Fulhorst et al., 1996). In order of decreasing strength of the reaction, reactivity was also observed with antibodies to Tamiami, Whitewater Arroyo, Latino, Pirital, and Flexal viruses (Fulhorst et al.,

1997). Comparison of partial NP sequences confirmed the similarities between Pichinde virus and the above lineage A viruses.

3.2 Pichinde as a Model System.

Unlike other members of the arenavirus family, Pichinde virus does not produce illness in humans (Mifune et al., 1971). Since it is safe for laboratory study and does not require high levels of containment, it has served as an invaluable model for the more virulent arenaviruses, and for principles which are characteristic of arenavirus infection in general.

The replication of Pichinde virus *in vitro* has been well-characterized. It produces a persistent, non-cytopathic infection in Vero and baby hamster kidney (BHK) cells (Young et al., 1986). These cell types synthesize abundant viral proteins, and produce high levels of infectious virus, yet exhibit no morphological changes (Shivaprakash et al., 1988; Young et al., 1986).

Since arenaviruses possess a tropism for cells of the reticuloendothelial system (Gonzalez et al., 1980; Murphy et al., 1977; Murphy et al., 1976), the effects of arenaviral infection on these cell types have been elucidated by analyses of the replication of Pichinde virus in monocytic and macrophage cells *in vitro*. Human monocytic cell lines induced to differentiate to macrophage-like cells by phorbol esters were shown to support Pichinde virus replication (Polyak et al., 1995; Polyak et al., 1991; Lewis et al., 1989). Growth in the monocytic line U937 was also enhanced by the presence of immune anti-Pichinde serum (Lewis et al., 1989). Although the significance of this to arenavirus infections *in vivo* is not known, antibody-mediated enhancement of replication in monocytic cells has been shown to play a role in the pathogenesis of Dengue hemorrhagic fever (O'Sullivan et al., 1994). Pre-treatment of the promonocytic leukemia cell line THP-1 with phorbol myristate acetate (PMA) enabled Pichinde virus

growth, and the stimulatory effects on viral replication were believed to involve protein kinase C activation, and transcriptional induction (Polyak et al., 1991). In addition, it was shown that human peripheral blood monocytes were also susceptible to infection with Pichinde virus, as evidenced by fluorescent antibody staining and by the immunoprecipitation of viral proteins from infected cells (Polyak et al., 1991).

The observation that Pichinde virus dramatically inhibited DNA synthesis in infected mouse peritoneal macrophages provided the first indication that arenavirus infection may disturb macrophage cell function (Friedlander et al., 1984). Since proliferation of macrophages has been thought to accompany the development of cell-mediated immunity, the inhibition of this function may impair recovery from infection.

3.3 Animal Models of Infection.

The value of Pichinde virus as a model has also been demonstrated in many *in vivo* systems, which have greatly elucidated such principles as: arenaviral persistence, the role of host genetic factors in susceptibility to lethal virus infections, and the pathogenesis of the virulent arenaviruses.

Studies performed at the time of its original isolation demonstrated that Pichinde virus produces a lethal infection following intracranial inoculation of neonatal mice (Trapido and Sanmartin, 1971). Adult mice, however, are refractory to infection, regardless of the route of inoculation. The susceptibility of neonatal mice to Pichinde virus appears to vary according to the strain of mouse. Instead of a lethal infection, certain strains, such as Balb/c, develop a persistent infection, whose features differ from those of an LCMV persistent infection (Wright et al., 1995). This system enabled the study of the pathophysiology of a persistent infection, and the role of the host genetic background in the outcome of viral infections, and provided a potential model for the study of arenaviral persistence in an unnatural host.

Studies of the replication of Pichinde virus in hamsters provided fundamental evidence of the reticuloendothelial tropism of arenaviruses (Murphy et al., 1977). Principle areas of viral involvement included the macrophages in the white pulp of the spleen, and the Kupffer cells of the liver, while lymphoid cells were not infected. In addition, the hamster model facilitated the investigation of host strain-specific differences in susceptibility to virus infection (Murphy et al., 1977; Buchmeier and Rawls, 1977). Hamsters of the random-bred LVG/LAK strain were susceptible to lethal infection within the first 6 days of life, but were resistant from 8 days of age onward (Buchmeier and Rawls, 1977). On the contrary, hamsters of the inbred MHA strain were highly susceptible throughout life, with rapid growth of the virus in kidneys, spleens, and brains of infected MHA animals. The fact that this growth occurred prior to the involvement of specific immunity, and that no mononuclear inflammatory lesions were observed in the tissues of MHA animals suggested that the sensitivity of the MHA strain to virus infection was related to an innate inability to control virus replication (Murphy et al., 1977). It was proposed that MHA hamsters possessed higher numbers of target cells, possibly cells mediating natural killer (NK) activity, and that this contributed to the fatal outcome (Gee et al., 1981).

Serial passage of a second Pichinde virus (Pichinde Munchique) in the spleens of guinea pigs resulted in an adapted strain (AdPic) which was uniformly lethal for strain 13 guinea pigs, and produced an illness similar to that observed in Lassa virus infection of monkeys and humans (Jahrling et al., 1981). The virulence of the adapted strain was associated with exuberant virus replication (Aronson et al., 1994). Relative to guinea pigs infected with the parental virus, significant increases in the number of infected peritoneal macrophages and splenic macrophages were detected in AdPic-infected guinea pigs. The guinea pig system provides a very useful model for the study of the pathogenesis of human arenaviral infections.

3.4 Temperature-Sensitive Mutants.

The availability of conditionally lethal mutants, such as those that are high temperature-intolerant, is an asset in the study of the stages of viral replication and virion morphogenesis. A series of *ts* mutants of Pichinde virus were generated by chemical mutagenesis of the *wt* parent (Shivaprakash et al., 1988; Vezza and Bishop, 1977). When grown at the non-permissive temperature, 39.5°C, the yields of each mutant were reduced by at least two log₁₀, relative to those obtained at the permissive temperature, 34°C (Shivaprakash et al., 1988). The mutants *ts1*, *ts2*, *ts3*, *ts5*, *ts10*, *ts11*, *ts12*, and *ts13* were first described by Vezza and Bishop (1977). When crossed, certain mutants recombined at a high frequency to form reassortant progeny with the *wt* phenotype. This enabled the mutants to be divided into two complementation groups. Those classified in Group I, which contained defects in the S segment (Veza et al., 1980), successfully complemented those classified in Group II, which contained defects in the L segment (Veza et al., 1980).

The additional *ts* mutants *ts274*, *ts454*, *ts488*, *ts538*, *ts908*, and *ts939* were generated by Shivaprakash et al. (1988) and characterized, along with the mutants described above. Based on the synthesis of RNA and immunoprecipitable proteins at the non-permissive temperature, 5 phenotypic patterns were observed. Cells infected with the mutants *ts538*, *ts1*, *ts3*, *ts5*, and *ts939* at the non-permissive temperature synthesized reduced amounts of NP protein, and undetectable amounts of GPC and L proteins, relative to those at the permissive temperature. Although all three proteins were detected in cells infected with *ts2*, they were present in reduced quantities at the non-permissive temperature. The decreased protein synthesis observed in these two groups was related to reduced levels of genomic and message RNA, suggesting defective transcriptase activity. The mutants *ts488* and *ts274* exhibited patterns of protein and RNA synthesis which resembled those of the *wt*. The nature of the *ts* defect in these viruses was

unclear. The fourth phenotypic group consisted of *ts11* and *ts454*, whose *ts* mutations had been localized to the S segment (Shivaprakash et al., 1988; Vezza et al., 1977). These two mutants synthesized reduced levels of protein in cells incubated at either the permissive or non-permissive temperatures. Finally, the mutants *ts13* and *ts908* synthesized RNA species which were identical in quantity and kinetics to those synthesized by the *wt*. However, there was an accumulation of GPC in cells incubated at the non-permissive temperature. Consequently, GPC was not translocated to the surface of infected cells, as evidenced by the failure to detect the mature glycoproteins by surface immunofluorescence (Shivaprakash et al., unpublished observations). Since *ts13* was previously placed in complementation group I by Vezza and Bishop (1977), these results further supported the presence of the *ts* mutation on an S segment gene, possibly GPC.

3.5 Identification of an Attenuated Mutant.

The study of viral glycoprotein processing and transport has been of primary interest in our laboratory. For this reason, the *ts* mutants *ts13* and *ts908*, which appeared defective in these processes, were obtained for further examination. The association of temperature-sensitivity with an attenuated phenotype has frequently been described. To investigate the phenotypes of *ts13* and *ts908* in this regard, their replication in adult mice was studied. Surprisingly, it was found that the growth of *ts13* was markedly restricted (Dr. K. Wright, unpublished observations). The results of a single representative experiment are presented in Table 2. It should be noted that, due to the established reticuloendothelial tropism of Pichinde virus, the spleen is the organ often assayed for viral replication, since a considerable proportion of the cells therein are macrophages (Junqueira et al., 1992; Abbas et al., 1991). As observed in Table 2, both the *wt* virus and *ts908* replicated in Balb/c mice, as evidenced by the detection of these two viruses at all time points, with peak levels observed in the spleen 72 hours post-inoculation (p.i.).

However, *ts13* was detected only at 24 hours p.i., and not at subsequent times. This restricted growth was also observed in C57 B6 mice (Dr. K. Wright, unpublished observations), indicating that this is not an MHC-restricted phenomenon. Thus, it was determined that *ts13* is attenuated for growth in adult mice. Although the degree to which *ts13* was attenuated for virulence could not be assessed in this model, its reduced growth may signify reduced pathogenic potential in models in which Pichinde virus infection is normally lethal. The studies to be described were undertaken in order to further characterize the behaviour of *ts13*, and to gain a greater understanding of the basis for its reduced growth, which will be referred to as attenuated growth.

Table 2: Initial analysis of the replication of *ts13* *in vivo**. (Dr. K. Wright, unpublished data).

Virus	24 hrs	48 hrs	72 hrs
wtDA	15 ⁺	94	107
ts908	94	89	876
ts13	29.3	<u>0</u>	<u>0</u>

* Balb/c mice, 4-6 weeks of age, were inoculated intraperitoneally with 5.0×10^7 pfu of the wild type, *wtDA*, or the mutants, *ts908*, and *ts13*, and their spleens examined for infectious virus 24, 48, and 72 hrs p.i..

⁺All data are expressed as the number of infectious centres (IC) per 10^6 cells.

HYPOTHESIS

As a result of genetic changes, Pichinde *ts13* has a reduced replicative ability in adult mice, compared to that of the *wt* virus. This results from reduced growth in certain tissues, and may involve particular cell types.

OBJECTIVES

Arenaviruses and their associated illnesses are a serious health concern in endemic regions, and new pathogens are continually emerging. Since the need for preventative measures is now apparent, it would be desirable to produce vaccines using attenuated arenaviruses, as these are effective and confer both humoral and cell-mediated immune protection. The identification of another attenuated arenavirus is therefore of great use, since it facilitates analyses of the mechanisms of arenavirus attenuation, and of the genetic changes necessary to attenuate growth and virulence. Such investigations are paramount to the development of appropriate vaccines. In view of employing *ts13* to further elucidate principles in attenuation among the arenaviruses, this work was undertaken with three objectives:

- 1) To characterize the growth of *ts13* both *in vivo* and *in vitro*, in order to identify potential tissues and cell types involved in limiting the growth of this mutant.
- 2) To determine a mechanism by which the growth of *ts13* is restricted *in vivo*.
- 3) To begin to identify genetic changes responsible for the attenuated phenotype.

MATERIALS AND METHODS

1. CELL LINES.

Cell cultures were incubated at 37°C in a water-jacketed incubator, in an atmosphere of 5% CO₂. Vero-CLL cells, as well as the murine fibroblast cell lines Balb/c C17 and C57-SV (provided by Jackson Laboratories, and described by Knowles et al., 1979) were grown in minimal essential medium (MEM) (Life Technologies, Grand Island, NY) supplemented with 5% fetal bovine serum (FBS) (Wisent), 2 mM L-glutamine (L-gln) (Life Technologies) and 1% Penicillin/Streptomycin. Baby Hamster Kidney (BHK21) cells were maintained in Dulbecco's Modified Eagle Medium (D-MEM) (Life Technologies) containing 5% FBS, and 5% tryptose-phosphate broth (TPB), in addition to L-gln. The murine macrophage cell line J744A.1 (American Type Culture Collection) was grown in D-MEM containing 10% FBS, plus L-gln.

2. VIRUSES.

The wild type (*wt*) virus used for these studies was provided by Dr. D. Auperin (Pfizer) and was designated *wtDA*. This virus has been described in sequencing studies in the literature (Auperin et al., 1984). The temperature-sensitive (*ts*) mutants *ts13* and *ts908* were obtained from Dr. W. Rawls (McMaster University, Hamilton, ON). For the preparation of virus stocks, BHK cells were grown in 75 cm² flasks (Corning) and infected with the appropriate virus at a multiplicity of infection (MOI) of 0.1. Inocula were adsorbed to cells at 33°C for the *ts* viruses, and at 37°C for the *wt*, in a volume of 3.0 ml of D-MEM plus 8% FBS, plus L-gln. Following incubation for 1 hour, inocula

were replaced with 20 ml of the same media and further incubated for 48 to 72 hours. At this time, virus was harvested by collecting supernatants, and centrifuging for 5 minutes at 300 x g, at room temperature, to remove cellular debris. Stocks were stored at -80°C.

3. VIRUS QUANTIFICATION.

Virus in stock solutions, sera, and tissue homogenates was quantified by plaque assay (Mifune et al., 1971). Briefly, serial ten-fold dilutions of virus samples were prepared in PBS containing 0.1% gelatin. Vero monolayers grown in 12-well plates (Falcon, Lincoln Park, NJ) were inoculated with 0.1 ml per well of each dilution. Duplicate wells were plated for each dilution. Virus was permitted to adsorb for 1 hour at 37°C. At this time, inocula were aspirated and 1.5 ml of an overlay were added to each well. The overlay consisted of a 1:1 mixture of 1.2% Noble Agar and 2 x 199 medium containing 10% FBS plus L-gln and antibiotics. Plates were incubated at 37°C for three days. Viral plaques were then visualized by flooding wells with 0.03% (w/v) neutral red in PBS, and incubating overnight at 37°C. Stocks of *ts* virus were titrated at 33°C and 39.5°C to verify the temperature sensitivity. The limit of detection of the plaque assay was approximately 10 to 30 plaque-forming units (pfu) per ml of culture supernatant, when plating samples from undilute to out to dilutions of 10⁻⁴. Walker et al. (1985) previously found that the limit of Pichinde virus detection in the spleen was 1 log PFU per gram of tissue.

Virus-infected cells were quantified as Infectious Centres. Suspensions of known numbers of infected cells (refer to Appendix B) were serially diluted, plated in

duplicate onto Vero monolayers, incubated, and stained to reveal plaques, as described above. Each plaque originated from one infected cell, and was counted as an infectious centre. Titres were expressed as the number of Infectious Centres (IC) per 10^6 cells. The sensitivity of this method was 1 IC per 10^6 cells.

4. MICE.

Female or male Balb/c mice, aged 4-6 weeks, were obtained from Charles River Laboratories (Montreal, PQ) and housed according to specifications of the animal care committee at The University of Ottawa. Animals were inoculated intraperitoneally (IP.) with either 4.0×10^7 or 8.0×10^7 of *ts13* or *wtDA*, in a volume of 0.3-0.5 ml per mouse.

4.1 Harvesting of Cells and Tissues.

a) Resident Peritoneal Cells.

Resident peritoneal cells (RPC) from normal and infected mice were harvested by peritoneal lavage. After mice were euthanized, peritoneal cavities were injected with 6 ml of PBS, which was removed after gentle massaging of the cavities. This process was repeated once. Unseparated cells were washed once with RPMI medium (Life Technologies) containing 10% FBS and 2 mM L-gln, re-suspended in 2 ml of medium, counted, and assayed for infectious centres, as described in the following section. For separation into plastic-adherent and non-adherent populations, the entire RPC suspension was permitted to adhere to 60-mm plastic dishes (Falcon) at 37°C in a volume of 4-5 ml. Following incubation for 2.5 hours, non-adherent cells were pipetted off. Dishes were washed 3 times with medium to remove additional non-adherent cells.

When the RPC were collected from infected mice, adherent cells were scraped into fresh medium using a rubber spatula. Both the adherent and non-adherent preparations were resuspended in 0.4 ml of medium, counted, and assayed for infectious centres. RPC derived from normal mice were infected *in vitro* at an MOI of 0.1 with either *ts13* or *wfDA*. Adherent cells were infected directly in the dishes, whereas non-adherent cells were infected in suspension.

b) Spleen, Liver, and Kidney.

Spleens, livers, or kidneys were removed aseptically and placed into 5 ml of complete RPMI medium. Each organ sample was adjusted to a concentration of 10% (w/v) in medium, and disrupted for 1-2 seconds using a small tissue homogenizer (Omni). Homogenates were frozen at -80°C prior to assay for virus.

c) Serum.

Blood was collected from anaesthetized infected mice by cardiac puncture. Samples were allowed to coagulate for 20 minutes at 37°C and were then centrifuged twice at $9000 \times g$ at 4°C to separate the clear serum from the cellular debris.

d) Mesenteric Lymph Nodes.

Mesenteric lymph nodes (MLN) were removed aseptically into 12 ml of complete RPMI medium. Approximately 3 lymph nodes per mouse were collected. Single cell suspensions were prepared by forcing MLN through wire screens. Suspensions were washed once and re-suspended in a volume of 0.4 ml. Cells were counted and assayed for infectious centres.

4.2 Temperature Monitoring.

To measure body temperature, an electronic temperature monitor fitted with a rectal probe was used (Physitemp Instruments). The temperatures of 5 mice were read 24 hours before and immediately prior to IP inoculation in order to establish a base-line body temperature, then prior to sacrifice, the temperatures of all mice were re-assessed.

5. RADIOIMMUNOPRECIPITATION AND SDS-PAGE ANALYSIS OF PICHINDE PROTEINS.

Vero cell monolayers were infected at an MOI of 0.1 with either *ts13* or *wtDA*, and at 48 hours p.i., the medium was replaced with methionine (met)- and cysteine (cys)-free MEM (Life Technologies) for 30 min at 37°C. Each dish was labeled with 60 µCi of a mixture of ³⁵S-Cell Labeling Mix (Amersham), in a volume of 1.0 ml, for 1.5 hrs at 37°C. Cells were washed once with cold PBS, and then lysed at 4°C with 1.0 ml of a lysis buffer, consisting of 20 mM Tris-HCl, pH 9.0, 137 mM NaCl, 1 mM CaCl₂, 0.5 mM MgCl₂, 10% (v/v) glycerol, 1% (v/v) NP-40, and 1 mM each phenylmethyl sulphonyl fluoride (PMSF) and benzamidine hydrochloride. Lysates were cleared of cellular debris by centrifugation for 5 minutes at 9000 x g at 4°C. A polyclonal Pichinde-specific rabbit antiserum (prepared by K. Wright) was added to each lysate at a final dilution of 1:70, and samples were incubated for 2.5 hours at 4°C on a rotating platform. One hundred µl of a 2.5% (w/v) suspension of ProteinA-Sepharose beads (Sigma Chemical Co., St. Louis, MO) were added to each sample. Samples were incubated for another 45 min at 4°C, then washed three times with a wash buffer consisting of 100 mM Tris-HCl, pH 9.0, and 500 mM LiCl. After the final wash, protein-antibody-ProteinA-bead complexes were suspended in 30 µl of sample buffer (125 mM Tris-HCl, pH 6.8,

2% (v/v) SDS, 2% (v/v) 2-mercaptoethanol, 20% (v/v) glycerol, and 0.05% (v/v) bromophenol blue), and subjected to electrophoresis through 8.5% SDS-polyacrylamide gels based on the system of Laemli (1970). Gels were fixed in 25% (v/v) methanol and 7.5% (v/v) acetic acid, soaked in Amplify (Amersham, Buckinghamshire, England) to enhance detection of radioactivity, dried, and exposed to X-ray film (Kodak, X-AR).

6. SEQUENCING OF THE NUCLEOPROTEIN OF TS13.

The sequence of the NP gene of *ts13* was generated from PCR amplicons of reverse-transcribed viral S RNA. RNA was prepared from *ts13* stocks as follows. One ml of stock (corresponding to $>2.0 \times 10^8$ pfu) was treated with 200 $\mu\text{g/ml}$ proteinase K and 1% SDS, for 30 min at 56°C. The sample was then extracted once with buffer saturated phenol (Life Technologies), once with phenol:chloroform, and once with chloroform. RNA was precipitated by adding 0.5 volumes of 7.5 M ammonium acetate (NH_4OAc), and 2.5 volumes of absolute ethanol and incubating overnight at -80°C. Samples were centrifuged for 30 min at 9000 x g at 4°C, washed with 75% ethanol, dried under vacuum, and re-suspended in 10 μl of diethyl pyrocarbonate (DEPC)-treated distilled H_2O .

2.5 μl of RNA were used as the template in a 25 μl reverse transcription reaction containing 1X reaction buffer (Life Technologies), 1.25 mM dNTP's, 10 mM DTT, 38.6 units Porcine RNA Guard (Pharmacia), 100 ng of primer 3'S-RNA (Appendix A) and 50 units of Expand Reverse Transcriptase (Boeringer-Mannheim Canada, Laval, QUE). The reaction was incubated overnight at 37°C, and 4 μl of the cDNA product was used for PCR amplification. The PCR reaction mixture contained 1X PCR buffer (Life

Technologies), 3 mM MgCl₂, 200 μM dNTP's, 100 ng each of primers 3'S-RNA and PIC-S-2 (Appendix A), and 2.5 units of Taq polymerase (Life Technologies). The profile of each cycle was 1 min at 94⁰C, 2 min at 48⁰C, and 3 min at 72⁰C. The amplicon was 2049 nucleotides in size and contained (approximately) 200 bases at the end of the GPC gene, the intergenic region, the entire coding sequence of NP and the 3' non-coding region of the S RNA.

PCR products were purified from 0.8% low-melting temperature agarose gels in 1X TAE buffer (40 mM Tris-HCl, pH 8.0, 0.11 % (v/v) glacial acetic acid, 1 mM EDTA). Bands were excised, melted at 65⁰C, and the DNA was eluted by adding an equal volume of a buffer containing 100 mM Tris-HCl, pH 8.0, 1 mM EDTA, and 40 mM NaCl. After heating at 65⁰C for 15 min, with occasional vortexing, the mixture was extracted once with buffer-saturated phenol, once with phenol:chloroform, and once with chloroform. DNA was precipitated for 1 hour at -20⁰C and re-suspended in 20 μl distilled H₂O.

Sequencing was performed in the automatic sequencing facility at the Biotechnology Research Institute of the University of Ottawa. Both strands of the template DNA were sequenced. The viral primers cNP-2, cNP-3, and cNP-4 (see Appendix A) were used to sequence the virus sense strand, whereas the primers vNP-5, vNP-6, and vNP-7 were used to sequence the complementary strand.

RESULTS

1. INITIAL STUDIES OF THE REPLICATION OF TS13 IN VIVO.

Following the discovery that the replication of *ts13* in adult mice was greatly restricted compared to that of the *wt* virus, *wtDA*, several experiments were performed in order to repeat this observation and to further characterize the behaviour of this attenuated virus *in vivo*. Since only the spleen had originally been studied, it was also of interest to examine virus replication in other tissues and organs. Four to six week-old Balb/c mice were inoculated intraperitoneally (IP) with 4.0×10^7 pfu of either *ts13* or *wtDA*. On the first, second, or third day post-inoculation (p.i.), two mice per virus were sacrificed, and their spleens, livers, and blood were collected. Tissue homogenates and sera were assayed for infectious virus by plaque assay. As observed in Table 3, infectious *ts13* was undetectable in the spleen. In contrast, the *wt* virus replicated considerably in the spleen, and was detectable at all time points examined, reaching maximal titres 48 hrs p.i.. These results confirm the marked attenuation of *ts13*. *Ts13* was also undetectable in serum, whereas *wtDA* was present at 2 of 3 time points. The levels of *wtDA* in the serum were greatest 24 hrs p.i.. Unexpectedly, neither virus was detected in the liver. The apparent absence of an alternate site of *ts13* replication, either in the liver or in the blood, suggests that this virus does not display an altered tropism relative to the *wt* virus. It should be noted that, in the initial studies, *ts13* retained its *ts* nature *in vitro* following growth in the mice.

Table 3. Detection of *ts13* in the spleen, and serum.

Tissue / Virus		Infectious virus (pfu) per g spleen or per ml serum		
		Day 1 (p.i.)	Day 2	Day 3
Spleen	<i>wtDA</i>	1.00×10^5	2.56×10^6	1.76×10^5
	<i>ts13</i>	0	0	0
Serum	<i>wtDA</i>	1.27×10^3	6.00×10^2	0
	<i>ts13</i>	0	0	0

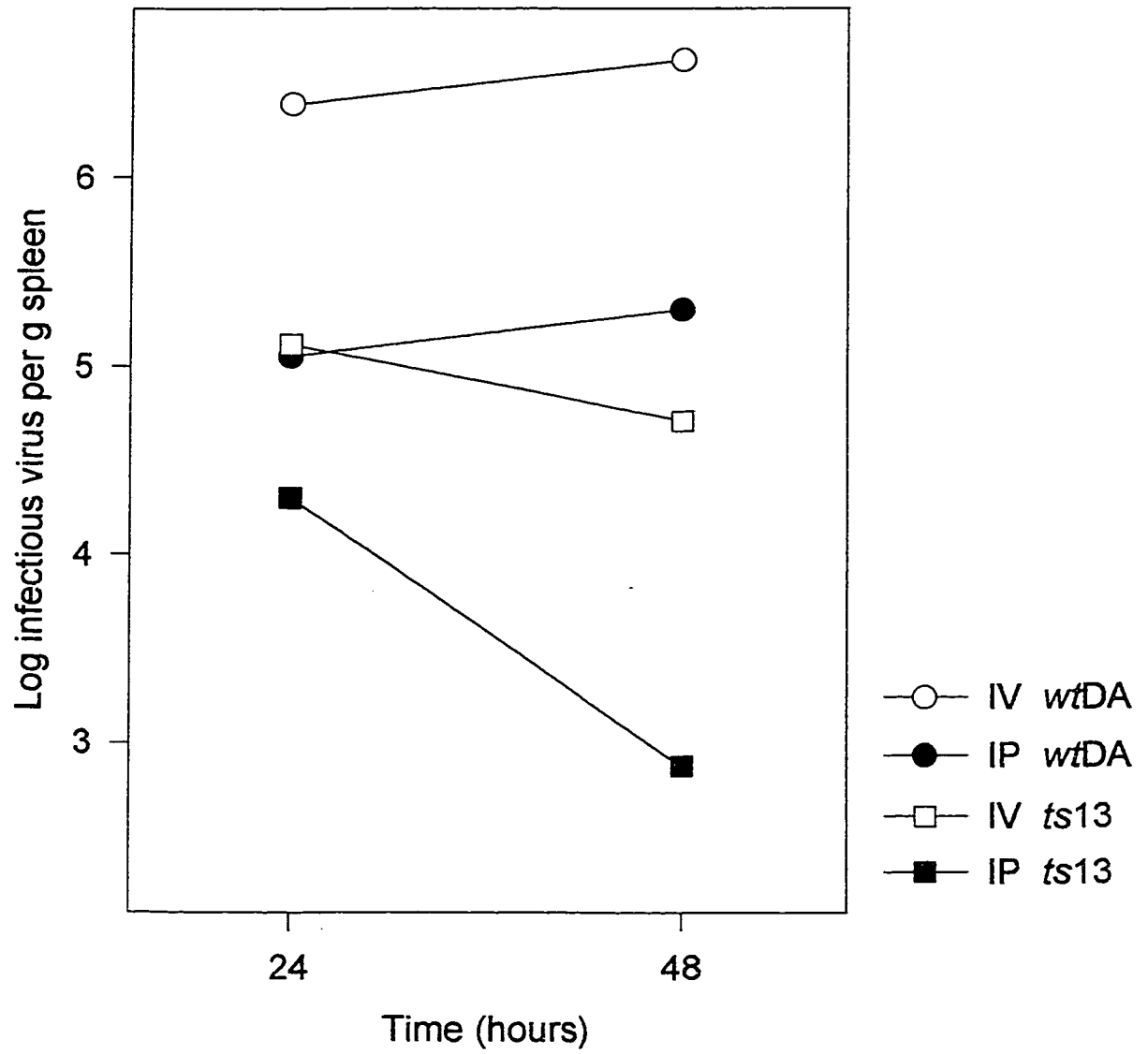
Balb/c mice at 4-6 weeks of age were inoculated intraperitoneally with 4.0×10^7 of either *wtDA* or *ts13*. Following infection for 1, 2, or 3 days, the spleens and sera of two mice per virus were harvested and pooled as described in Materials and Methods, and assayed for infectious virus by plaque assay on Vero cell monolayers. Above are the results of a single experiment.

2. EFFECT OF THE ROUTE AND SIZE OF INOCULATION ON THE DETECTION OF TS13 IN VIVO.

It was surprising that *ts13* was completely undetectable in the previous experiment, given that this mutant had been detected consistently in earlier experiments, albeit at low levels. The animals had been inoculated by the IP route, whereas those used in the initial experiments were inoculated by the intravenous (IV) route. Since IV inoculations are thought to allow a more direct delivery of virus inocula to tissues and organs, it was suspected that the failure to detect *ts13* in the previous experiment was related to the IP route of inoculation. To verify this, IP and IV inoculations were performed in parallel, and the size of the virus inoculum was increased to 8.0×10^7 pfu. Balb/c mice were infected with either *ts13* or *wtDA*, and at 24 and 48 hrs p.i., two mice per virus were sacrificed for each route of inoculation. Spleens were then harvested, pooled, and assayed for infectious virus. As depicted in Figure 1, both viruses were detectable following IP and IV inoculations. However, levels of virus detected in the spleen following IV inoculation were 0.80 to 2.00 \log_{10} greater than those obtained following IP inoculation. This emphasizes the importance of the route of inoculation in the detection of Pichinde virus in tissues. With the increased virus dose, regardless of the route of inoculation, the levels of *ts13* in the spleen were 0.80 to 2.40 \log_{10} lower than those of *wtDA*.

3. REPLICATION OF TS13 IN ORGANS AND CELLS OF THE IMMUNE SYSTEM.

Figure 1. Comparison of Pichinde virus replication in the spleen following intraperitoneal or intravenous inoculation. Balb/c mice were inoculated with 8×10^7 pfu of *w/DA* or *ts13*, either by the intraperitoneal (IP) or the intravenous (IV) route. At twenty-four and 48 hours post-inoculation (hrs p.i.), two mice per virus were sacrificed for each route of inoculation, and their spleens were harvested and homogenized, as described in Materials and Methods. Spleen homogenates were assayed for infectious virus by plaque assay on Vero cell monolayers. Data are expressed as the natural log of the infectious virus per g of spleen tissue. The results presented were obtained from a single experiment.



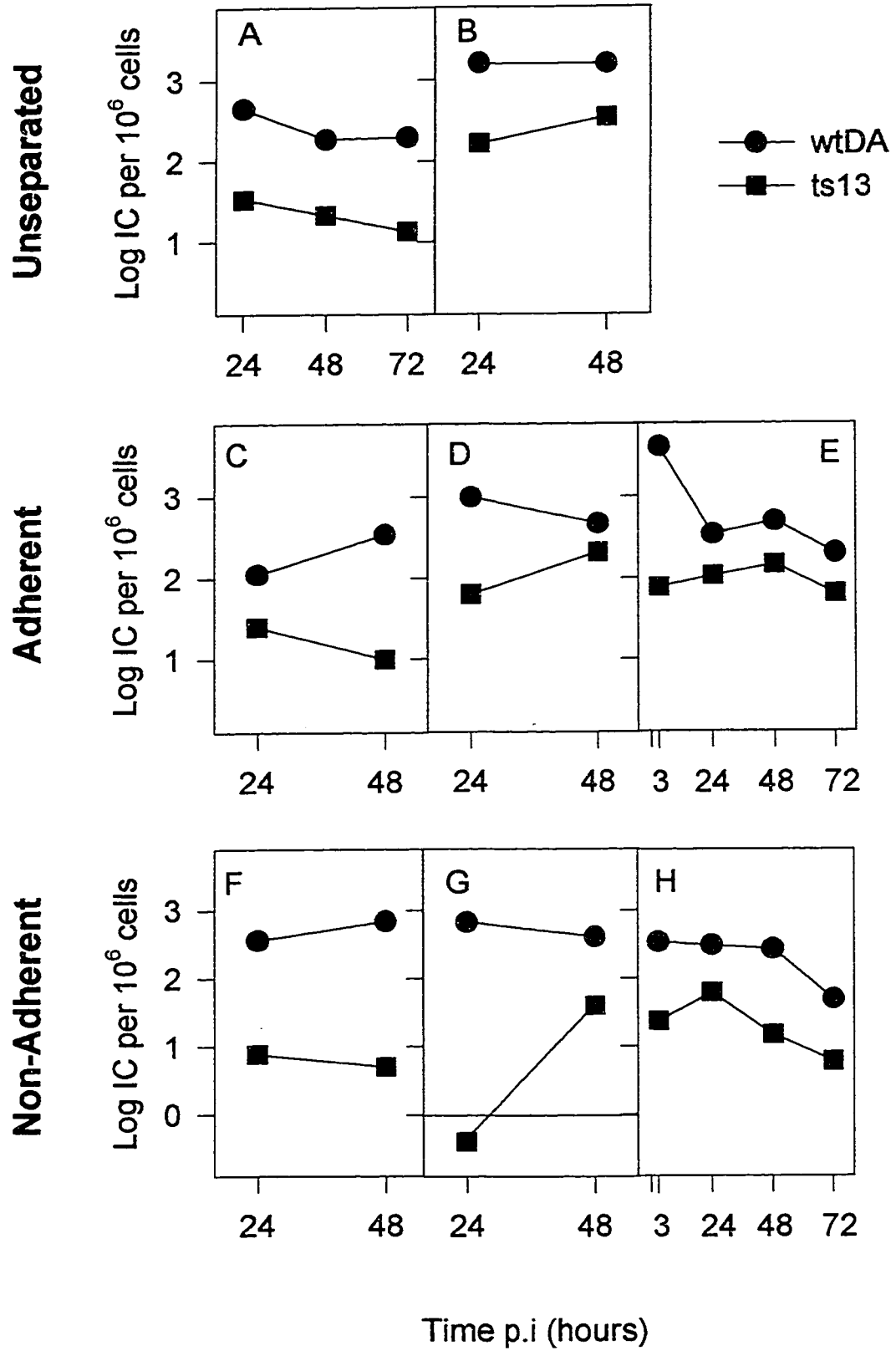
To further characterize the replication of *ts13 in vivo*, after IP inoculation, the resident peritoneal cells (RPC), which are located near the site of inoculation, were assayed for virus. The replication of *ts13* in these cell types, as well as in several other tissue types, is described subsequently. It should be noted that *ts13* retained its *ts* phenotype following growth *in vivo*, verifying that no selection for revertants had occurred.

3.1 Replication in the Resident Peritoneal Cells.

Adult Balb/c mice were inoculated IP with 8.0×10^7 pfu of either *ts13* or *wtDA*. In a series of 3 experiments, RPC were collected from mice, by peritoneal lavage, at 24, 48, and 72 hrs p.i.. Firstly, virus-infected cells in unfractionated RPC were enumerated by infectious centres assays. Panels A and B of Figure 2 each display the results of one experiment. The number of infectious centres (IC) recovered from *ts13*-infected mice was consistently reduced relative to that recovered from *wtDA*-infected mice.

Since the RPC are a mixture of several cell types, the behaviour of the viruses in different cell populations was examined by fractionating RPC on the basis of plastic adherence prior to assay for virus infection. It is generally accepted that the plastic-adherent population is comprised almost exclusively of macrophages (King et al., 1990; Wood et al., 1979), whereas the non-adherent population is a mixture of monocytes and lymphocytes (Plasman and Vray, 1993). It is of note that the conditions utilized for generating the RPC populations were very similar to those described by King et al. (1990). Figure 2, C to H, presents the results of three separate experiments. Panels C, D, E each represent one experiment, in which adherent RPC populations were assayed.

Figure 2. Replication of Pichinde virus in the resident peritoneal cells (RPC) of infected Balb/c mice. Animals were inoculated intraperitoneally (IP) with 8.0×10^7 of either *wfDA* or *ts13*. At 24, 48, or 72 hrs p.i., mice were sacrificed and their RPC removed by peritoneal lavage, as described in Materials and Methods. RPC from two to three mice were pooled for each time point. RPC were assayed for infectious centres, either immediately following collection, or following fractionation into plastic-adherent and non-adherent populations. Results are expressed as the common log of the infectious centres (IC) per 10^6 cells. Points situated beneath the horizontal line represent virus which was below the limit of detection, which was 1 IC per 10^6 cells ($\log_{10}=0.00$) for the RPC. Presented are the results of four separate experiments, arranged according to the type of cell preparation assayed, namely unseparated, adherent or non-adherent. Each panel displays the results of a single experiment. One experiment was comprised only of the results shown in panel A. Panels B, D, and G correspond to the same experiment, as do panels C and F, and panels E and H.



The numbers of IC in the corresponding non-adherent fractions are shown in panels F, G, and H, respectively. In both populations, the numbers of *ts13*-associated IC was consistently reduced relative to that of *wtDA*. As shown in Figure 2, the numbers of *ts13* IC observed in the non-adherent population were considerably lower than those of the *wt*, and, at one 24 p.i. time point, were below the limit of detection (1 IC per 10^6 cells). When mean log reductions were compared in the adherent and non-adherent populations, it can be seen that, at 24 and 48 hrs p.i., *ts13* demonstrated the greatest reduction in the non-adherent population (Table 4). In unseparated RPC, the mean magnitude of the decrease in *ts13* IC was approximately $1.00 \log_{10}$ for each time point, as summarized in Table 4. Analyses of numbers of IC in all RPC populations suggest that, in addition to the spleen, the growth of *ts13* is also highly restricted in cell types such as the RPC.

In one experiment, the relative numbers of IC of the 2 viruses were assessed very early in the infection by harvesting RPC 3 hrs p.i., and fractionating. It was proposed that a 3 hr time point would permit sufficient time for the viruses to adsorb to target cells, yet would be prior to the onset of viral replication. The numbers of *ts13*- and *wtDA*-infected cells at this time would therefore be indicative of their relative abilities to bind to and enter RPC. As observed in panels E and H of Figure 2, the reduction in the number of *ts13*-associated IC relative to those of *wtDA* was considerable in both cell populations at 3 hrs p.i.. The magnitudes of these differences were $1.73 \log_{10}$ and $1.16 \log_{10}$ in the adherent and non-adherent cells, respectively (Table 4). These results suggest that the attachment of *ts13* to target cells near the site of inoculation and its

Table 4. Comparison of the numbers of *ts13* IC with those of *wfDA* in the RPC of infected mice.

	Mean log reduction in <i>ts13</i> IC compared to <i>wfDA</i> IC*			
	3 hrs (p.i.)	24 hrs	48 hrs	72 hrs
Unseparated	NA ⁺	1.06 (n=2) ⁰	0.80 (n=2)	1.17 (n=1)
Adherent	1.73 (n=1)	0.79 ± 0.36 (n=3)	0.76 ± 0.53 (n=4)	0.51 (n=1)
Non-Adherent	1.16 (n=1)	1.73 ± 1.07 (n=3)	1.29 ± 0.60 (n=4)	0.62 (n=2)

* In each experiment shown in Figure 2, the numbers of *ts13*-associated IC were consistently reduced compared to those of *wfDA*. For each panel presented in this Figure, the differences in the log IC per 10⁶ cells of *ts13* relative to those of *wfDA* were calculated, for every time point. Displayed here are the means of these differences ± standard deviation, in cases where data from at least 3 experiments were included.

⁰ Shown in brackets are the number of measurements reflected by the mean.

⁺ Not assayed.

subsequent entry may be impaired compared to the *wt*. The differences between viruses were similar in both the adherent and non-adherent cells at this time point, indicating that *ts13* may be restricted at early stages in both cell types.

3.2 Replication in the Mesenteric Lymph Nodes.

The previous experiments revealed that the growth of *ts13* was limited in tissues rich in macrophages and lymphocytes, as evidenced by the observations in the non-adherent population of RPC. To determine whether *ts13* was similarly restricted in lymph nodes, the mesenteric lymph nodes (MLN) of infected animals were assayed for infection by virus. Murine lymph nodes are composed predominantly of lymphocytes (D. Conte, personal communication). Figure 3 shows the results of 3 separate assays, as depicted in Panels A, B, and C, respectively. As was observed in the RPC, the numbers of IC in the MLN from *ts13*-inoculated mice were markedly reduced relative to those in MLN from *wt*-inoculated mice. *Ts13* was often below the limit of detection. When *ts13* was detectable, the number of IC recovered was 1.00 \log_{10} lower than the number of *wtDA*-associated infectious centres at 24 hrs p.i., and as much as 2.45 \log_{10} at later times. Table 5 summarizes the mean reduction in the numbers of *ts13*-associated IC in the MLN, relative to those of *wtDA*. The mean differences in the numbers of IC for the two viruses ranged from 0.98 \log_{10} to 2.45 \log_{10} , and were greatest 48 hrs p.i.. This was consistent with the time at which the differences in virus levels were maximal in the spleen.

The kinetics of *wtDA* replication in the MLN were similar to those observed in the spleen (Table 3). Maximum numbers of IC were achieved 48 to 72 hrs p.i., with

Figure 3. Detection of *ts13* in the Mesenteric Lymph Nodes (MLN) of infected mice. Animals were inoculated as stated previously (Figures 2, 3, 4), and lymph nodes were harvested and crushed into single cell suspensions. Cells were processed as described in Materials and Methods, and were assayed for infectious centres. Presented are the results of 3 separate experiments, each panel representing one experiment. In panels B and C, results are expressed as the common log of the IC per 10^6 cells. In the experiment depicted in panel A, cells had been ruptured prior to the assay by freezing them at -80°C . Thus, the results for this panel are expressed as the common log of the infectious virus released from 10^6 cells. Horizontal lines indicate the limit of virus detection, which was 1 IC per 10^6 cells ($\log_{10}=0.00$). Panel A represents the same experiment as panels B, D, and G of Figure 2, and panel B of Figure 3. Panel B represents the same experiment as panels E and H of Figure 2 and panel C of Figure 3. Panel C represents the same experiment as panel D of Figure 3.

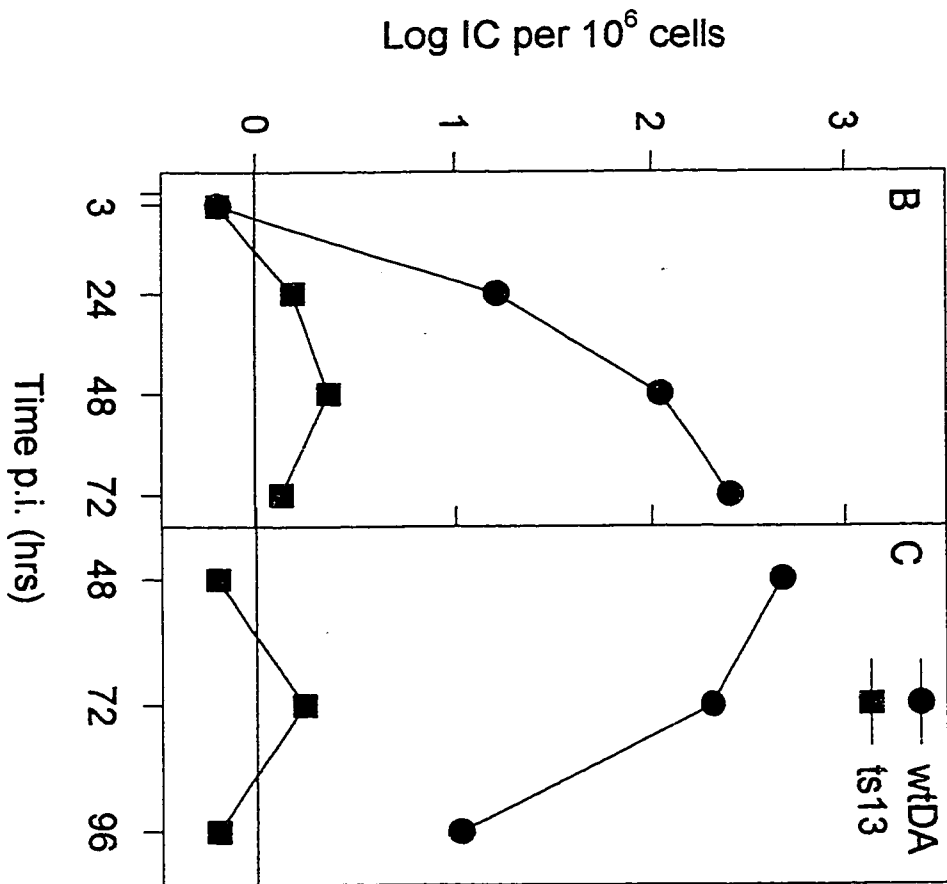
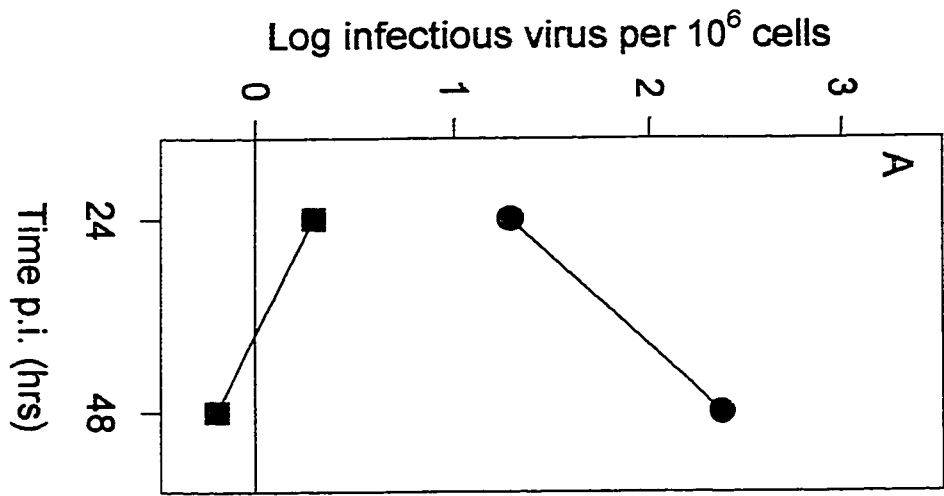


Table 5. Comparison of the relative levels of *ts13* and *wtDA* in the spleens and relative numbers of *ts13* and *wtDA*-associated IC in the MLN of infected mice.

	Mean log reduction in the levels of <i>ts13</i> or number of <i>ts13</i> IC relative to <i>wtDA</i> *			
Tissue	24 hrs	48 hrs	72 hrs	96 hrs
Spleen	1.82 ± 1.54 (n=3) ⁰	2.61 ± 0.35 (n=4)	2.08 (n=2)	1.19 (n=1)
MLN	0.98 ± 0.04 (n=3)	2.45 ± 0.57 (n=4)	2.04 ± 0.25 (n=3)	1.02 (n=1)

* For each panel presented in Figures 3 and 4, the levels of *ts13* in the spleen or numbers of *ts13*-associated IC in the MLN were markedly reduced relative to those of *wtDA*. The differences between the log pfu per g tissue (spleen) or log IC per 10⁶ cells (MLN) for *ts13* compared to those of *wtDA* were calculated, for every time point. Presented here are the means of these differences ± standard deviation where data from at least 3 experiments was included. It should be noted that when *ts13* was undetectable in the MLN, its levels were assigned a value corresponding to the limit of detection (1 IC per 10⁶ cells, or log₁₀ = 0), for purposes of comparison with the *wt*.

⁰ The number of measurements reflected by each mean are indicated in parentheses.

numbers decreasing at 96 p.i.. In panel A of Figure 3, the numbers of IC for *ts13* decreased between 24 and 48 hrs p.i., indicating an absence of measurable replication. In the experiments depicted in panels B and C, numbers of *ts13* IC increased steadily until 48 or 72 hrs p.i., suggestive of a low level of replication. Nevertheless, the replication of *ts13* was always considerably less vigorous than that of the *wt*. From these 3 experiments, it was clear that the replication of *ts13* is also limited in tissues containing a large proportion of lymphocytes.

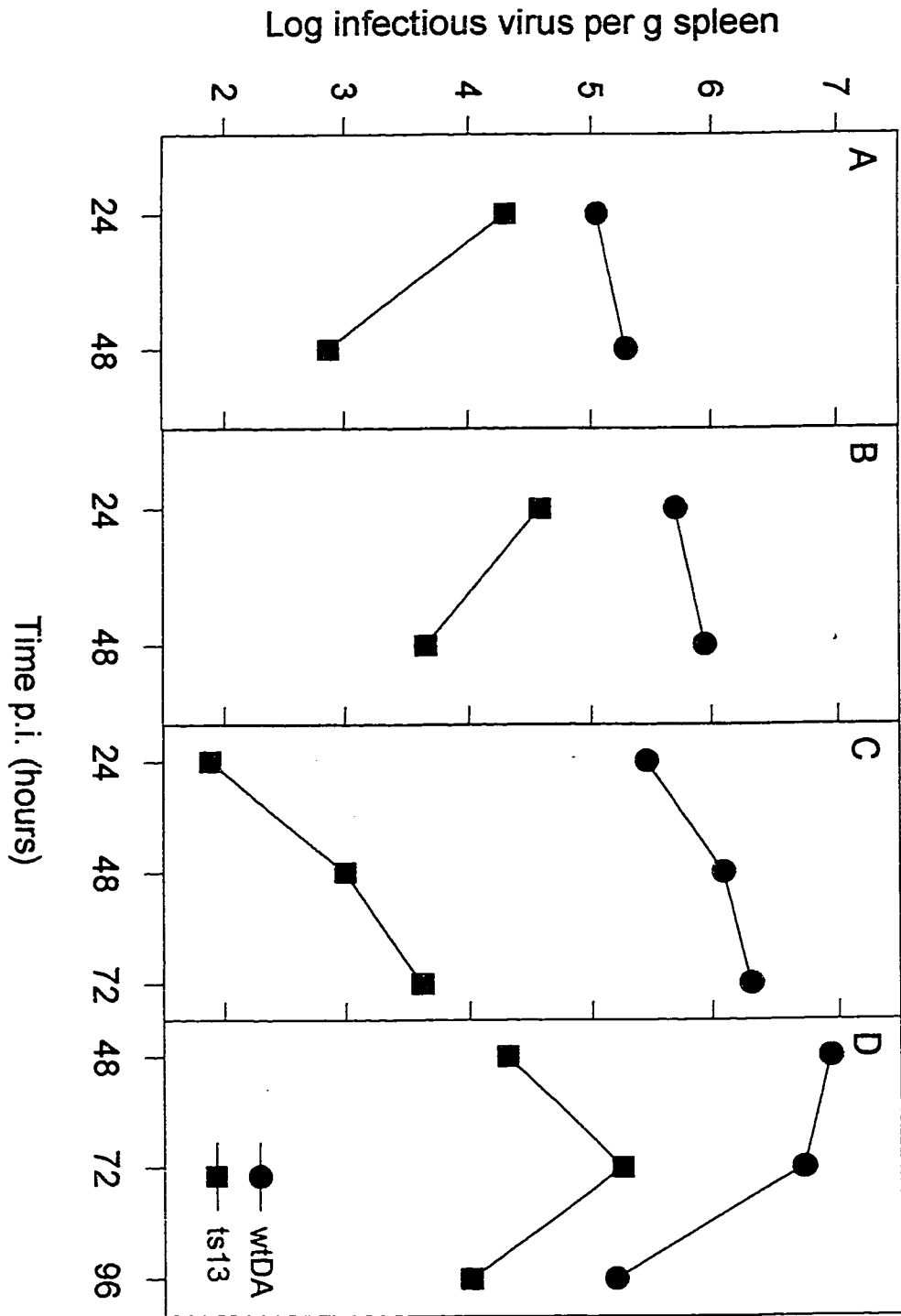
3.3 Replication in the Spleen, Liver and Kidney.

In the same series of experiments, virus in other sites was also examined. Spleens, livers, and kidneys were harvested at various 24 hr intervals p.i., and assayed for infectious virus. In these experiments, *ts13* was consistently detected in spleens in each of the four separate experiments presented (Figure 4). Compared to *wtDA*, the replication of *ts13* in the spleen was highly restricted, and the extent of this restriction was again greater than in the RPC. Table 5 presents a summary of the mean reductions in the levels of *ts13* relative to those of the *wt* at time points out to 96 hrs. Although this table combines the results of at least 4 different experiments, not all time points were examined in every experiment. The mean differences in the levels of the two viruses were at least 1.8 log₁₀ for most time points, and were greatest 48 hrs p.i. (2.61 log₁₀). Neither virus was detected in the livers or kidneys of infected mice.

Titres of *wtDA* regularly increased between 24 and 48 hrs p.i., and peaked 48 to 72 hrs p.i. (Figure 4). Levels began to decline after 72 hrs. *Ts13*, however, often failed

Figure 4. Replication of Pichinde virus in the spleens of infected Balb/c mice.

Animals were inoculated with 8.0×10^7 pfu of *ts13* or *w/DA* by the IP route. At various 24 hr intervals p.i., animals were sacrificed and the spleens of 2-3 mice per virus were pooled. Spleens were then homogenized and assayed for infectious virus on Vero monolayers. Results are expressed as the common log of the infectious virus per g of spleen tissue. Each panel presents the results of a single experiment. Panel A represents the same experiment as panels C and F of the previous figure, whereas panel B corresponds to panels B, D, and G of that figure, and panel C corresponds to panels E and H. The experiment corresponding to panel D is not represented in the previous figure.



to replicate, as observed in Figure 4, A and B. This reflected the behaviour in the corresponding RPC (Figure 2, C and F). When replication of *ts13* was observed (Figure 4, C and D), it was highly impaired and failed to attain the levels demonstrated by the *wt*.

4. EFFECTS OF TEMPERATURE ON THE REPLICATION OF TS13 IN VITRO.

One possibility to explain the limited growth of *ts13* was that the mouse body temperature, 37°C (Smeenk et al., 1996), was non-permissive for *ts13* replication. To examine this, an efficiency of plating (EOP) assay was performed, in which stocks of *ts13* and *wtDA* were titrated at 33°C, 37°C, and 39.5°C. The EOP was expressed as the ratio of the titre at 39.5°C to the titre at 37°C. As indicated in Table 6A, the titre of *ts13* at 37°C was not reduced compared to that at 33°C, which is considered a permissive temperature for replication of the *ts* mutants. Only at 39.5°C, the non-permissive temperature for the *ts* mutants, was the titre of *ts13* decreased. The EOP for this virus was 0.06. The *wt* virus demonstrated no reduction in titre at the non-permissive temperature, as reflected by the EOP of 1.05. These results showed that the growth of *ts13* is not impaired by temperatures up to 37°C.

The *ts* phenotype was further verified by assaying the replication of *ts13* at both the permissive (33°C) and the non-permissive temperature. To ensure that stocks of *ts13* prepared in our laboratory were identical to those previously characterized (Shivaprakash et al., 1988; Veza and Bishop, 1977), a 1996 stock and a stock from 1978 (provided by D. Harnish, McMaster University) were both used to infect BHK cells. Infected cells were incubated at 33°C or 39.5°C for 72 hrs, at which time

Table 6. Titration and growth of ts13 at various temperatures.

A. Efficiency of Plating (EOP) Assay.

	Infectious virus (pfu) per ml virus stock			
	33°C	37°C	39.5°C	EOP*
<i>wiDA</i>	1.53×10^7	3.76×10^7	3.96×10^7	1.05
<i>ts13</i>	3.20×10^6	4.16×10^6	2.38×10^5	0.06

B. Growth of *ts13* at the non-permissive temperature.

	Infectious virus per ml culture supernatant [†]			
	33°C		39.5°C	
Growth temperature:	33°C		39.5°C	
Plating temperature:	37°C	39.5°C	37°C	39.5°C
<i>wiDA</i>	2.50×10^6	2.86×10^6	4.40×10^7	5.67×10^7
<i>ts13</i> - 1978	3.81×10^4	0	1.50×10^3	0
<i>ts13</i> - 1996	1.45×10^6	3.42×10^4	2.00×10^4	750

* The EOP ratio is the ratio of the titre at 39.5°C and the titre at 37°C.

† Cultures of BHK cells were infected at an MOI of 0.1. Following infection for 72 hrs at 33°C or 39.5°C, culture supernatants were collected and assayed for virus on Vero monolayers. Shown are the results of a single experiment.

supernatants were assayed for virus. The titres of *ts13* produced at the non-permissive temperature were reduced by 1.40 log₁₀ (*ts13*-1978) and 1.86 log₁₀ (*ts13*-1996) compared to those produced at 33°C (Table 6B). In contrast, *wlDA* demonstrated no *ts* phenotype, as the quantities of virus produced at 39.5°C were almost identical to those produced at 33°C. The progeny *wlDA* also retained the non-*ts* phenotype.

The possibility existed that Pichinde virus infection of mice elevated the body temperature to a non-permissive level, thereby restricting the replication of *ts13*. To investigate this, body temperatures were monitored during one of the previous experiments. To establish a baseline, the temperatures of 5 mice were measured both 24 hrs before and immediately prior to inoculation. Temperatures were monitored again prior to sacrifice. The baseline temperature was approximately 35°C. At 3 hrs p.i., temperatures increased to 35.8 and 36.2°C for animals infected with *wlDA* and *ts13* respectively (data not shown). This increase may have been due to inoculation-induced stress. Between 24 and 72 hrs p.i., the average body temperature of *wlDA*-infected mice ranged from 34.9 to 35.4°C. That of *ts13*-infected mice ranged from 35.5 to 36.3°C. These data demonstrated that, during infection with *wlDA* or *ts13*, the mouse body temperature did not increase beyond permissive levels.

5. EXAMINATION OF THE GROWTH OF TS13 IN MURINE CELLS IN VITRO.

5.1 Replication in a Murine Macrophage Cell Line.

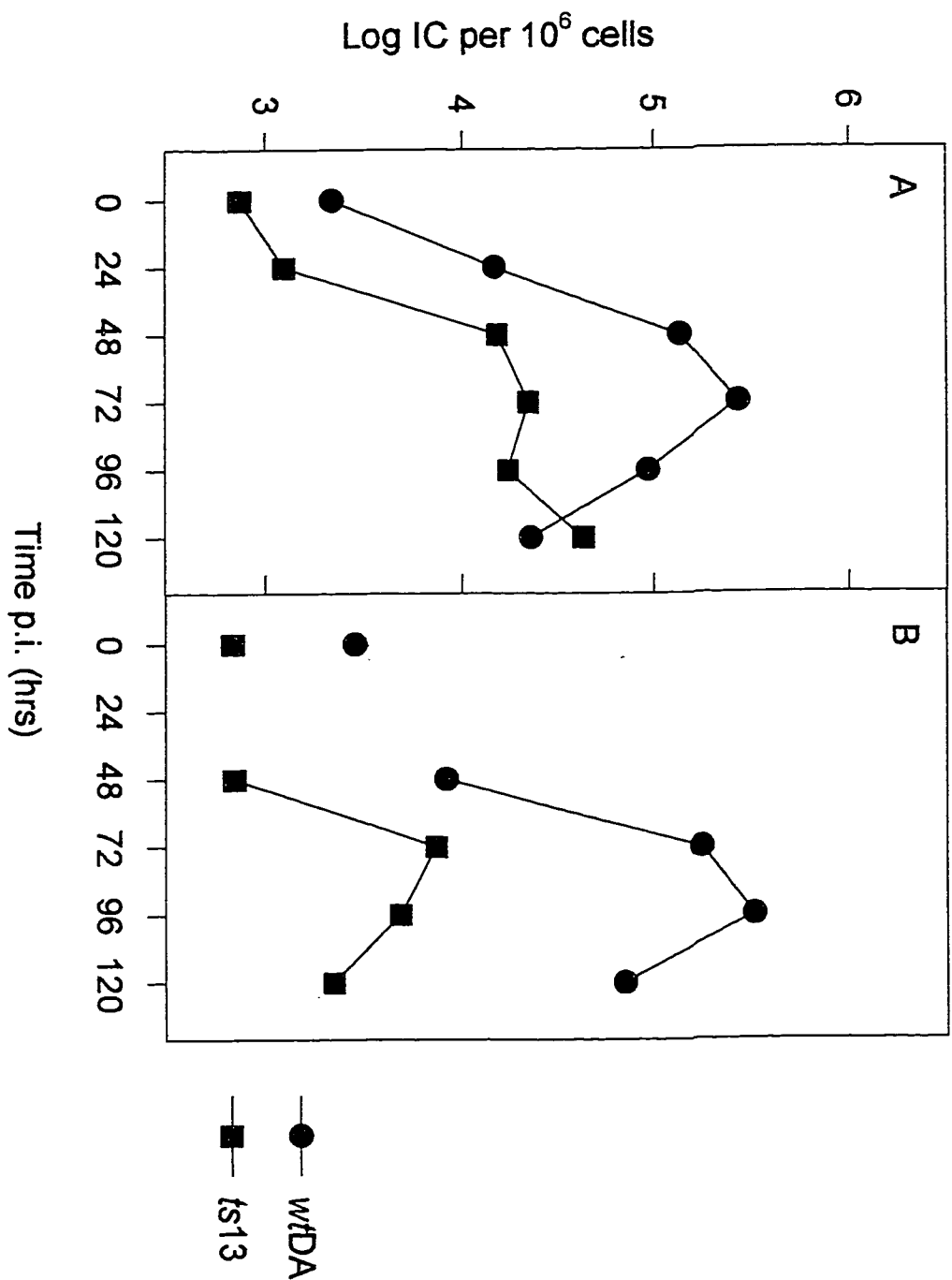
Macrophages and monocytes are of great importance in the establishment of arenaviral infections *in vivo* (Gonzalez et al., 1980; Murphy et al., 1977; Ambrosio et al.,

1988). The restricted growth of *ts13* *in vivo* in cells and tissues enriched for macrophages suggested that an inability to replicate efficiently in these cell types might play a role in the attenuated phenotype. This was further explored by studying the replication of *ts13* in a pure macrophage population, the Balb/c cell line J744A.1. The replication of *ts13* in these cells was compared to that of *wtDA*. After infection at an MOI of 0.1 or 0.05, cells were harvested and assayed for infectious centres or cultured for 24 to 120 hrs and then assayed. Figure 5 portrays the results of 2 experiments, of a total of 4 experiments. In both experiments, the levels of cell-associated *ts13* were consistently reduced by 1.00 to 1.50 \log_{10} relative to those of *wtDA*, consistent with the results observed in the RPC *in vivo*. The numbers of IC of both viruses peaked at approximately 72 hrs p.i., showing that the kinetics of *ts13* replication in the macrophages were similar to those of the *wt*. Interestingly, differences in the levels of the two viruses were observed at the 0 hr time point (immediately after the 1 hour adsorption period). This result suggests possible differences in the abilities of the two viruses to bind to and enter macrophages.

5.2 Replication in Resident Peritoneal Cells *In Vitro*.

Since *ts13* displayed reduced replication in a murine macrophage cell line, it was next necessary to examine its behaviour in a macrophage-enriched cell population which was more physiologically relevant. This was accomplished by studying replication after *in vitro* infection of RPC harvested from uninfected mice. Adherent cells and non-adherent cells, were infected with *ts13* or *wtDA* at an MOI of 0.1, and assayed for infectious centres either immediately after the adsorption period or following culture for

Figure 5. Analysis of the replication of ts13 in murine macrophages. The J744A.1 macrophage cell line was infected in suspension with *ts13*, or *wlDA*, and incubated for 1 hour at 37°C. Cells were then washed once and resuspended in complete RPMI medium (see Materials and Methods) at a concentration of 1.5×10^6 cells per ml. Aliquots of 3.0×10^6 cells per well were seeded into 6-well plates and cultured at 37°C. Following culture for various 24-hour intervals, cells were harvested by gentle scraping, adjusted to a concentration of 5.0×10^6 cells per ml, and assayed for infectious centres. Aliquots of cells were also assayed immediately after infection, designated as 0 hrs p.i.. Depicted in panels A and B are the results of two separate experiments, in which macrophages were infected at an MOI of 0.1 and 0.05, respectively. Data are presented as the common log of the IC per 10^6 cells.



24 to 96 hrs. The replication of *ts13* in both the adherent and non-adherent populations was consistently reduced relative to that of *wtDA*. Table 7 lists the mean reduction in the numbers of cells infected with *ts13* relative to those infected with the *wt*, for every time point. In the adherent cells, the mean differences in the numbers of IC were similar to those determined for the RPC *in vivo* (Table 7, Table 4). However, differences in the non-adherent population *in vitro* were less striking than in the corresponding population *in vivo* (Table 7, Table 4).

5.3 Replication in Murine Fibroblast Cell Lines.

Although reduced growth in macrophages was proposed to be involved in the attenuated phenotype of *ts13*, the possibility of a generalized host-range restriction had not been discounted. To determine whether the reduced growth *in vivo* resulted from an inability to replicate efficiently within murine cells, the replication of *ts13* in continuous fibroblast cell lines of murine origin was studied. The cell lines Balb/c C17 (H-2^d) and C57-SV (H-2^b) were infected at an MOI of 0.1 with either *wtDA* or *ts13*. Twenty-four, 48, or 72 hrs p.i., culture supernatants were harvested and assayed for infectious virus. BHK cells, which are used for the propagation of virus stocks, were infected as a control. Figure 6 describes the results of multiple experiments, performed in each cell line. Panel A demonstrates that the replication of *ts13* in Balb/c cells was not reduced relative to the replication of the *wt* virus in these cells. The replication of *ts13* in these murine cells was also not reduced relative to that in BHK cells, which were included as a control, since they are used for preparing viral stocks. Similar results were obtained in C57-SV cells (panels B and C). In C57 cells cultured at 37^oC (panel B), the levels of

Table 7. Summary of the differences in the numbers of *ts13*- and *wtDA*-infected cells in RPC infected *in vitro*.

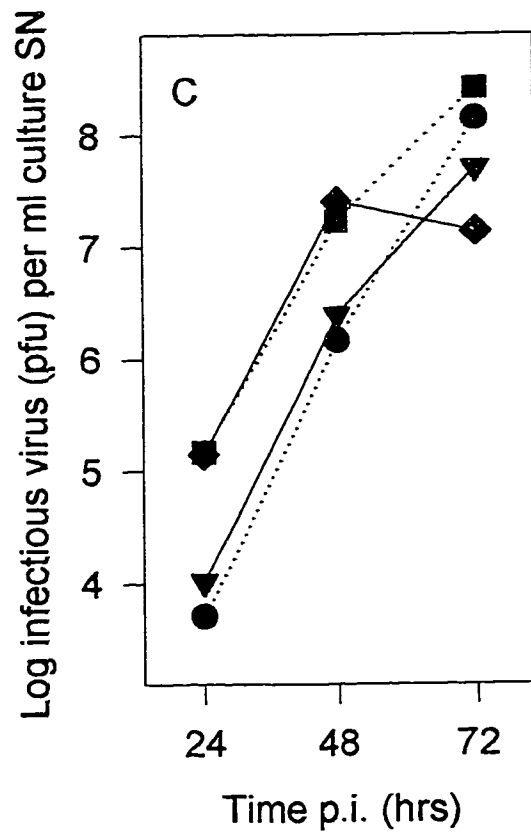
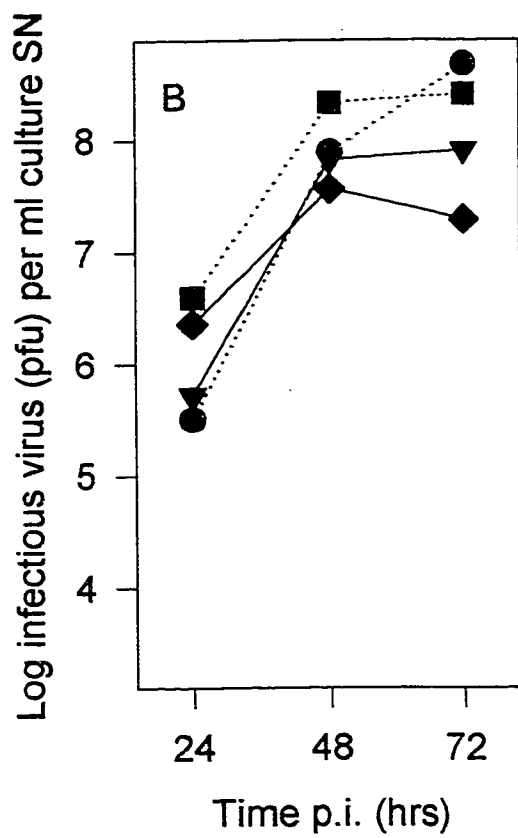
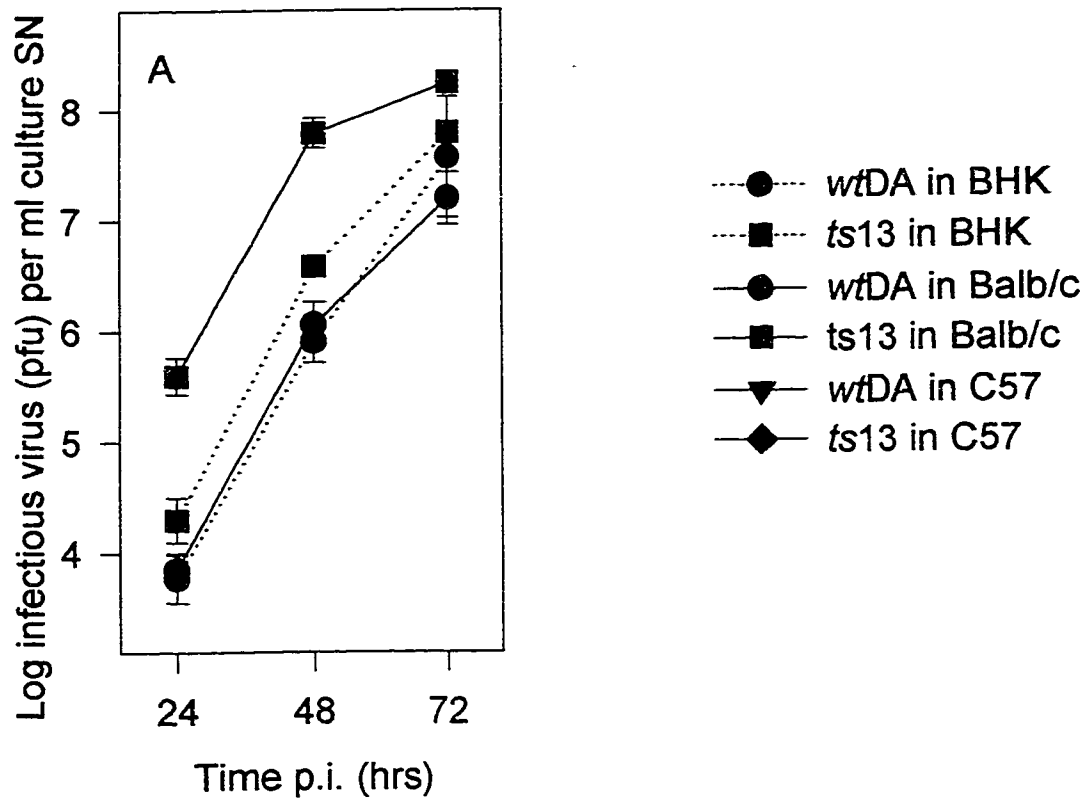
	Mean log reduction in <i>ts13</i> IC relative to those of <i>wtDA</i> *				
	0 hrs	24 hrs	48 hrs	72 hrs	96 hrs
Adherent	0.73 (n=3) (SD=0.65)♦	1.33 (n=2)	0.70 (n=3) (SD=0.25)	0.66 (n=2)	0.42 (n=2)
Non-Adherent	0.37 (n=3) (SD=0.30)	0.99 (n=2)	0.84 (n=3) (SD=0.17)	0.45 (n=1)	0.46 (n=2)

* For three separate experiments in which RPC were infected *in vitro*, the differences in the log IC per 10^6 cells for *ts13* relative to that of *wtDA* were calculated, for every time point. The means of these differences are presented here. Numbers in parentheses indicate the number of measurements for each mean.

♦ Standard deviation is given when the number of measurements is 3.

Figure 6. Growth of *ts13* in continuous fibroblast cell lines of murine origin.

Confluent monolayers of Balb/c C17 or C57-SV (Knowles et al., 1979) cells were infected at an MOI of 0.1 with either *ts13* or *wiDA*. BHK monolayers were infected as a control. Unless otherwise indicated, infections were performed at 37°C. Following incubation for 24, 48, or 72 hrs, culture supernatants were collected and assayed for infectious virus. Values are expressed as the common log of the infectious virus per ml of culture supernatant. Panel A displays a composite of three separate experiments using Balb/c C17 cells. The standard deviation is indicated by the error bars spanning each data point. Panels B and C each represent a separate experiment performed in C57-SV cells. In these two experiments, infected cells were incubated at 37°C and 33°C, respectively.



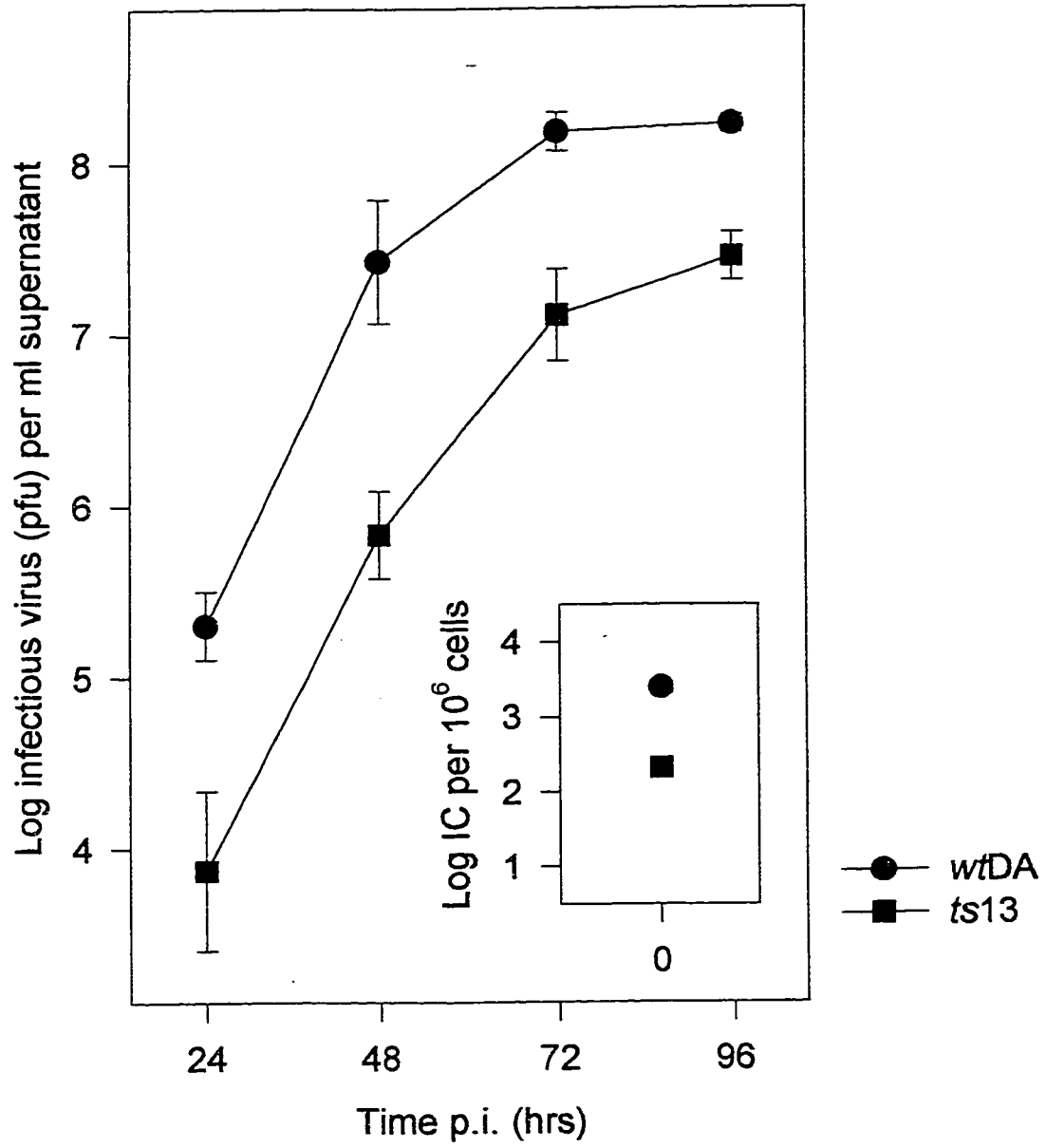
ts13 were not markedly reduced compared to those of the *wt* in the murine cells. When considered in combination, the results of these experiments imply that murine cells are permissive for the growth of *ts13*.

5.4 Replication in Primary Murine Embryonic Fibroblasts.

The murine cell lines Balb/c and C57-SV supported *ts13* replication as competently as did the control BHK cells. Nevertheless, these highly-passaged continuous cell lines may not have accurately reflected the behaviour of *ts13* in fibroblasts *in vivo*. For this reason, the replication of *ts13* in primary cells, which are expected to be more similar to those *in vivo*, was studied. Primary fibroblasts derived from embryonic mice (Balb/c X 129 F1), were kindly provided by N. Abraham (Ottawa Regional Cancer Centre). At the fourth *in vitro* passage following explantation, cells were infected at an MOI of 0.1 with *ts13* or *wtDA*. Following incubation for 24 to 96 hrs, culture supernatants were assayed for infectious virus. The combined results of two experiments are presented in Figure 7. The quantities of *ts13* released into the culture medium were generally reduced by at least 1.00 log₁₀, compared to those of the *wt* virus. The greatest difference in the levels of the two viruses was 1.60 log₁₀, on average, and was observed 48 hrs p.i. The two viruses displayed similar kinetics. Immediately after adsorption, the quantity of cell-associated *ts13* was approximately 1.00 log₁₀ lower than that of *wtDA* (Figure 7, inset). This again indicated that *ts13* may be impaired in its adsorption to target cells. The results of the replication in primary cells contradicted those obtained in the murine cell lines. This may reflect differences between primary and transformed lines, or differences between embryonic and adult cells.

Figure 7. Replication of *ts13* in primary murine embryonic fibroblasts.

Fibroblasts obtained from embryonic mice were infected at the fourth passage with either *ts13* or *wtDA*, at an MOI of 0.1. Following culture at 37°C for the indicated lengths of time, culture supernatants were collected and assayed for infectious virus. The relative levels of virus immediately following infection (0 hrs) were assessed by detaching cells with trypsin after the adsorption period, and assaying for infectious centres. Values are given as the common log of the infectious virus (pfu) per ml culture supernatant for the 24 to 96 hr time points, and as the common log of the IC per 10⁶ cells for the 0 hr time point. Each value represents the mean of two separate experiments. The standard deviation is indicated by the error bars spanning each data point.



6. GENETIC ANALYSIS OF THE S SEGMENT OF TS13.

Once *ts13* had been characterized, both *in vivo* and *in vitro*, it was of interest to identify genetic changes potentially responsible for the attenuated phenotype. It was determined by Vezza et al. (1980) that the *ts* defect maps to the S RNA segment. Although the mutations responsible for the attenuated phenotype may be different, sequence analysis of *ts13* was begun with this segment.

6.1 Glycoprotein Precursor (GPC).

The GPC gene of *ts13* had been previously cloned and sequenced (K. Wright, unpublished observations). It was discovered that there is a single nucleotide change in the GPC of *ts13*, relative to *wtDA* (Auperin et al., 1984). As listed in Table 8, this mutation converts an A residue to a G, resulting in an Asn to Ser substitution at residue 132 of the GP-1 portion of the GPC protein. This amino acid change eliminates a potential site of N-linked glycosylation, which consists of the residues Asn-132, Ile-133, and Ser-134 in the *wt* virus. To determine whether this site is utilized, the relative sizes of the GPC proteins of *ts13* and *wtDA* were examined, since the loss of a glycosylation site would produce a smaller protein for *ts13*. Viral proteins were radioimmunoprecipitated from infected Vero cells using a polyclonal Pichinde-specific rabbit antiserum. Immunoprecipitates were analyzed by SDS-PAGE. It was consistently observed that the GPC of *ts13* migrated more rapidly than that of *wtDA*. A representative autoradiogram is presented in Figure 8. This altered migration suggested that the *ts13* GPC was smaller in size, consistent with the loss of a single N-linked

Table 8. Sequence differences in the S segment of *ts13* relative to that of *wtDA*.

Gene / Region	Nucleotide ⁺	Amino acid [#]	Nucleotide change [◆]	Amino acid change [◆]
GPC*	446	132	A → G	Asn → Ser (AAU → AGU)
NP	2158	393	G → A	silent mutation (AUC → AUU)
	2278	353	G → A	silent mutation (CUC → CUU)
	2513	275	A → G	Ile → Thr (AUA → ACA)
Intergenic region	1627	/	A → C	/

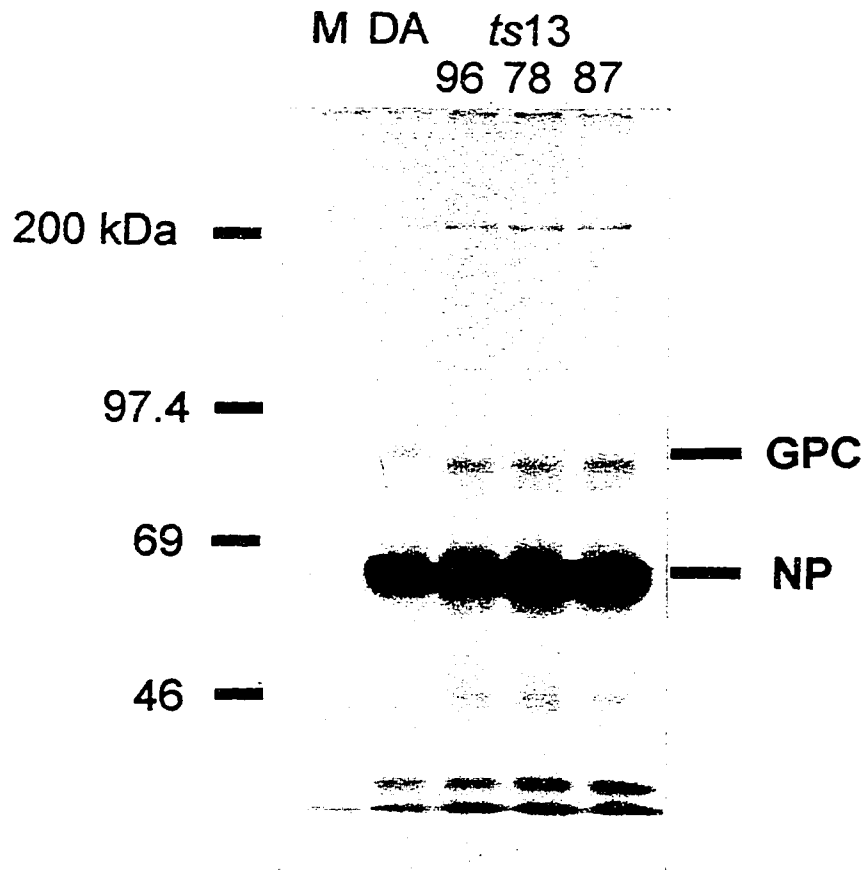
* The sequence of the GPC gene of *ts13* was determined previously (Dr. K. Wright, unpublished observations). The GenBank Accession number is U77602.

⁺ Nucleotide positions are listed relative to the 5' end of the viral RNA. Since the NP is encoded in the negative sense, nucleotide positions nearest the 3' end of the viral RNA are closest to the 5' end of the NP mRNA.

[#] Amino acid positions are listed relative to the amino-terminus of the particular protein.

[◆] The *wt* nucleotide or amino acid is given to the left of the arrow; the *ts13* sequence is given to the right. Codons are presented in the mRNA sense, which is identical to the viral sense for the GPC gene and complementary to the viral sense for the NP gene.

Figure 8. Effect of a Mutation in a Potential N-Linked Glycosylation Site on the Migration of the ts13 GPC. Vero cell monolayers were infected at an MOI of 0.1 with either *ts13* or *wtDA*. Three different stocks of *ts13* were used. Those prepared in 1978 and 1987, respectively, were provided by D. Harnish and were passaged once in our laboratory prior to the experiment. The 1978 stock is considered to be the original *ts13* stock. The *ts13* prepared in 1996 was generated from our laboratory stocks. Infected cells were incubated at 37°C for 48 hrs, at which time they were metabolically labeled with ³⁵S. Viral proteins were immunoprecipitated from cell lysates using a polyclonal Pichinde-specific rabbit anti-serum and electrophoresed on SDS-polyacrylamide gels. Lane M contained immunoprecipitates from uninfected cells. The sizes of the molecular size standards are given to the left. The long dashes indicates the positions of the GPC and NP proteins.



glycosylation site. Since this finding had not been reported in the original characterization of *ts13* (Shivaprakash et al., 1988), two earlier stocks of *ts13*, generated in 1978 and 1987, respectively, were obtained and assayed. It was discovered that these stocks also produced a more rapidly-migrating GPC. These analyses suggested that the potential N-linked glycosylation site is ordinarily used, and that a mutation removing this site was stably maintained in *ts13* stocks.

6.2 Nucleoprotein (NP).

Ts13-derived PCR products containing the entire NP gene, the intergenic region and 200 bp of the GPC gene were sequenced on both strands using the primers listed in Appendix A. Three nucleotide mutations were identified in the NP gene of the 1996 stock of *ts13* relative to the sequence published for the NP of *wtDA* (Auperin et al., 1984). These are described in Table 8. All changes were verified by sequencing relevant sections of the NP gene of *wtDA*. The first mutation converted an A at nucleotide 2513 of the viral RNA to a G. This resulted in a Ile to Thr substitution at amino acid 275 of the NP protein, thereby replacing a non-polar residue with a polar one. The other two mutations were both G to A substitutions. Occurring at nucleotides 2158 and 2278 respectively, each was silent and resulted in no amino acid change.

6.3 Intergenic Region.

Sequence analyses were also extended to the intergenic region between the GPC and NP genes, a region which extends between nucleotides 1564 and 1650 for Pichindevirus (Auperin et al., 1984). As listed in Table 8, an A to C substitution occurred at nucleotide 1627 of *ts13*, compared to *wtDA*. This mutation was located in

the putative stem-loop structure, at a position containing an A-G mismatch in the *wt* virus. Replacement of the A with a C results in proper base-pairing at this site, and may contribute to a tighter secondary structure in the *ts13* intergenic region.

DISCUSSION

A mutant of Pichinde virus, initially described on the basis of its temperature-sensitive (*ts*) phenotype, demonstrated restricted replication in adult mice. Similar behaviour was previously demonstrated in C57 Bl6 mice, whose haplotype is H-2^b, rather than H-2^d of Balb/c Cl7, suggesting that the phenomenon is not MHC-restricted (Dr. K. Wright, unpublished observations). Given that few attenuated arenaviruses had previously been described, this discovery was intriguing and provided a new opportunity to examine mechanisms in arenavirus attenuation. The work described in this communication was undertaken in order to further characterize the mouse-attenuated mutant *ts13* and to elucidate the basis for the reduced growth *in vivo*.

These results demonstrated that, in infected mice, *ts13* replicated in the same tissues and organs as the *wt* virus, namely the RPC, spleen, and MLN, and not the liver or kidney. The replication of *ts13* was typically reduced by at least 1 log₁₀ in all tissues assayed. In the adherent RPC, the mean reduction in the numbers of IC for *ts13* compared to those of *wtDA* were 0.51-0.79 log₁₀ for most time points. In other tissues, the differences were even greater, and were consistently at least 1 log₁₀ in the non-adherent RPC, 1.00-2.00 log₁₀ in the MLN, and as high as 2.61 log₁₀ in the spleen. Whereas these differences were maximal approximately 24 hrs p.i. in the RPC, they peaked 48 hrs p.i. in the spleen, which paralleled the time of maximal differences in the MLN. It was surprising that neither *ts13* nor *wtDA* were detected in the liver or kidneys, because both of these tissues were shown to harbour virus in the hamster model described by Murphy et al. (1977). Infections of mice with Pichinde virus may be less

efficient than in the natural hosts, and viruses may replicate in only a few organs or tissues. Therefore, Pichinde virus may not have spread beyond the primary lymphoid tissues, such as the spleen and lymph nodes, in the animals in this study, resulting in an absence of virus in the liver and kidneys. Alternately, Pichinde virus may have spread successfully to these organs, but may have been incapable of infecting the cells therein. It is also possible that a higher viral inoculum would be required in order to detect virus in these sites, or that virus would only be detectable if much later time points were assayed.

When a lower virus inoculum was delivered IP, *ts13* was undetectable *in vivo* compared to *wtDA*. This could be overcome both by increasing the inoculum size and by delivering the virus IV. This route of inoculation increased the delivery of virus to the spleen by 1.00-2.00 log₁₀, relative to the IP route. Nevertheless, marked differences in the levels of *ts13* and *wtDA* were apparent regardless of the route of inoculation.

The peak numbers of cells infected by *wtDA* in the MLN were similar to those in the RPC. Replication of this virus was also vigorous in the spleen, where levels approaching 1x10⁷ pfu per g of tissue were generated. In the experiments described, the kinetics of the growth of *wtDA* was relatively consistent, with maximal titres observed 24 to 48 hrs p.i. in the RPC, and 48 to 72 hrs p.i. in the MLN and spleen. This suggests that, in infected mice, early replication occurs in the RPC, whereas secondary replication occurs in the spleen and MLN, since IC and infectious virus peak later in these tissues. The kinetics of *ts13* were less consistent. In experiments where *ts13* did replicate, however, maximal titres occurred at times which coincided with those of the *wt*. Nevertheless, *ts13* was consistently present in greatly reduced quantities, compared to

the *wt* virus, and when replication was observed, it was markedly reduced relative to the *wt*.

With both viruses, considerable variation in titre was observed from experiment to experiment, especially in the RPC and spleen. Since this rendered statistical analyses difficult, it was not possible to determine whether the observed differences in the quantities of *ts13* relative to those of *wtDA* were significant. Regardless, the data presented demonstrate that the general behaviour of *ts13 in vivo* follows a distinct trend. Since *ts13* was restricted or undetectable in all cells and tissues examined, it can be concluded from these cumulative experiments that this mutant does not exhibit an altered tropism compared to the *wt* virus.

Some attenuated arenaviruses have previously been reported. Certain of these viruses are attenuated by nature. Among these are the African arenaviruses Mopeia, Mobala, and Ippy, and South American arenaviruses such as Tacaribe, and Tamiami. The prototype Pichinde strain An3739 is naturally attenuated for virulence in inbred strain 13 guinea pigs (Aronson et al., 1994; Jahrling et al., 1981). Whereas IP inoculation with AdPic, a strain adapted for virulence in inbred guinea pigs, resulted in 100% mortality, inoculation with an equivalent dose of the prototype virus killed only 15% of animals (Aronson et al., 1994). Serial passages in cultured cells have resulted in the production of the attenuated Junin virus strains XJCl3 (Guerrero et al., 1969, cited in Contigiani et al., 1993) and Candid#1 (Barrera Oro et al., 1985), which is the only current arenaviral vaccine candidate. Attenuated strains of Machupo virus produced by cell-culture adaptation were unstable, however, and reverted to virulence (Peters et al., 1987). Persistent infection of L cell cultures with LCMV gave rise to an attenuated

variant which protected mice against a lethal intracerebral challenge with the *wt* virus (Bruns et al., 1997). Like *ts13*, the attenuated Junin virus strain C167 was produced by chemical mutagenesis (Scolaro et al., 1989). These two viruses share the property of reduced growth in mice, and are both temperature-sensitive.

There have been frequent accounts of attenuation arising from *ts* mutations which prevent the mutant virus from replicating at the body temperature of the host animal (Richman and Murphy, 1979). The results of the efficiency of plating experiment demonstrated that *ts13* replicates at the mouse body temperature, 37°C (Smeenk et al., 1996), as efficiently as at 33°C, which is a permissive temperature. This suggested that the attenuated phenotype of *ts13* does not result from a restrictive effect of the normal body temperature. Following IP inoculation, the rectal temperatures of infected mice did not increase beyond permissive levels, indicating that the reduced growth of *ts13 in vivo* is probably not due to an increase in body temperature as a consequence of virus infection. However, the possibility of a localized temperature increase in the peritoneal cavity was not addressed. This increase could arise from the induction of pyrogenic inflammatory mediators such as TNF α following infection of the resident cells after an IP inoculation. For this reason, it would be of interest to compare quantities of this monokine in the RPC of uninfected, *ts13*-infected, and *wtDA*-infected mice. The patterns of TNF α production in inbred strain 13 guinea pigs infected with the avirulent, prototype PIC3739 strain of Pichinde virus were consistent with the idea that locally secreted TNF α produced early after infection is beneficial (Aronson et al., 1995). In contrast, aberrant regulation of TNF α production might be involved in the extreme

pathology of infection with the guinea-pig virulent strain AdPIC (Aronson et al., 1995). In guinea pigs infected with this virus, extremely elevated levels of TNF α mRNA were detected in the spleen and these levels persisted until the time of death. In addition, serum TNF α could be measured in these guinea pigs, but could not be detected in animals infected with the avirulent virus. A role for the RPC as a possible source of TNF α was suggested by the observations that infection with either virus strain primed peritoneal macrophages to produce TNF α when stimulated with LPS *in vitro* (Aronson et al., 1995).

The replication of ts13 in the murine fibroblast cell lines Balb/c Cl7 and C57-SV was not reduced compared to that in the control BHK cells. Further, its replication was also not reduced relative to that of wtDA, in either cell type. These findings indicated that murine cells are permissive for ts13, and that the attenuated phenotype is probably not due to a generalized inability to infect murine cells efficiently.

It should be emphasized, however, that these fibroblasts are continuous cell lines, which arise by repeated passage, leading to eventual transformation (Freshney, 1983). Such cells acquire considerable mutations, and may adopt new phenotypes. In theory, they may also lose certain characteristics, such as the ability to limit viral replication. It follows that the vigorous replication of ts13 observed in the continuous murine fibroblasts may not have been indicative of its behaviour in fibroblasts *in vivo*. It was therefore of interest to study the replication of ts13 in primary murine fibroblasts, or fibroblasts immediately derived from mice, since these are considered to permit virus replication to a similar extent as fibroblasts *in vivo*. The primary cells used were actually

derived from embryonic mice, rather than adults. The quantities of *ts13* released from these cells were at least 1.00 log₁₀ lower than those released by *wtDA*-infected cells. However, since these cells were embryonic, rather than adult, it is not certain whether the reduced growth of *ts13* therein was related to a general restriction in all murine cells, or to the immaturity of these cells. Given that the outcome of Pichinde virus infection in mice may be very different depending on the age of the animal (Wright et al., 1995), the replication of *ts13* in embryo-derived cells may not reflect that in the cells from the adult. It will next be necessary to examine the replication of *ts13* in primary fibroblasts derived from adult tissues.

The behaviour of *ts13* in macrophage cell types was a major focus of these studies, given the known tropism of Pichinde virus for these cells, and their importance in establishing arenavirus infections (Ambrosio et al., 1990; Murphy et al., 1977). This behaviour was first assessed *in vivo* by examining the growth in macrophage-enriched cells, namely the RPC, which are located at the site of virus inoculation. Both viruses were consistently detected in these cells, which are comprised of 70% macrophages (Plasman and Vray, 1993), suggesting that even the mutant is macrophage tropic. However, the replication of *ts13* was highly restricted. The plastic-adherent population of RPC is more highly enriched for macrophages, as previously shown by Wood et al. (1979). In this population, there was a consistent reduction in the numbers of *ts13*-associated IC, compared to those for *wtDA*, indicating that reduced replication in these cells may play a role in the attenuated growth. This was further supported by studies of the growth of *ts13* in the Balb/c macrophage cell line J744A.1. Regardless of the MOI, the replication of *ts13* in these cultured macrophages was restricted compared to that of

*wt*DA. The extent of this restriction was reflected by a 1.00-1.50 log₁₀ reduction in the numbers of *ts13*-associated IC, relative to those of the *wt*, which was consistent with the observations made in the RPC *in vivo*. The behaviour of the other *ts* mutant, *ts908*, was found to be more similar to that of the *wt* (Dr. K. Wright, unpublished data), confirming that the intriguing behaviour of *ts13* is unique to this virus.

Consistent mean reductions in the numbers of *ts13* IC were also observed in RPC infected *in vitro*, although these were generally less striking than *in vivo*, particularly in the non-adherent population. In the adherent population, these differences were more similar to those observed in the corresponding population *in vivo*. This confirms that the growth of *ts13 in vitro* is also impaired in macrophages which have not been subjected to selective factors imposed in the generation of continuous cell lines. Thus, reduced growth in the macrophages and monocytes, such as the RPC, may constitute one aspect of the overall restriction of *ts13*. However, if this was the sole factor involved, a similar degree of growth restriction might be observed in the spleen and MLN. Since the detection of *ts13* was dramatically reduced in these tissues, compared to the *wt*, it is likely that additional restrictive elements are involved.

The involvement of macrophages in arenavirus attenuation has been previously documented. Laguens et al. (1983) isolated macrophages and dendritic cells from the spleens of guinea pigs infected with either *wt* Junin virus, or its attenuated derivative, XJCl₃. It was found that both cell types harboured the *wt* parental virus, whereas the attenuated virus was detected only in the dendritic cells, suggestive of an inability of the XJCl₃ strain to infect macrophages. The two widely differing virulence phenotypes were attributed to this difference in target cells.

The guinea pig-virulent AdPIC strain of Pichinde virus exhibited increased replication in macrophages (Aronson et al., 1994). The numbers of infected peritoneal macrophages harvested from AdPIC-infected guinea pigs were 1.00-2.00 log₁₀ greater than those from guinea pigs infected with the attenuated An3739 strain. The pathology of AdPIC-infection was partially attributed to increased activation of macrophages (Aronson et al., 1994). Since these are the major TNF α -producing cells (Abbas et al., 1991), it is possible that increased replication of AdPIC in these cells impacted on TNF α regulation, enabling TNF α to have more systemic effects. In the experiments presented, neither virus was virulent, but the magnitude of the differences in growth was similar to those observed in the guinea pig model. It would be interesting to observe whether *wtDA* and *ts13* would demonstrate differences in virulence in a model system, such as neonatal mice, where the *wt* virus causes disease, and whether TNF α plays a role. Additionally, it would be useful to compare the TNF α production in *ts13*-infected macrophages, in order to determine whether this is further reduced compared to that in macrophages infected with the *wt*, which is the avirulent virus in the guinea pig model.

Thus, reduced growth in macrophages may be an important element contributing to attenuation of New World arenaviruses, and a property which should be taken into consideration in the development of additional attenuated strains.

Although lymphocytes are not known to be a principle target in arenavirus infections (Murphy et al., 1977), there is evidence that Pichinde virus is capable of replication in these cell types. Murine lymph nodes have been shown to support replication, as have murine thymocytes (Walker et al., 1985; Dr. K. Wright, unpublished

observations). Both of these tissues are highly enriched for T lymphocytes (Fung-Leung et al., 1994; Abbas et al., 1991). In the spleen and lymph nodes of MHA hamsters, cells demonstrating natural killer (NK) activity were shown to be targets for Pichinde virus infection, which provided additional evidence of an ability of the virus to replicate in certain lymphocyte populations (Gee et al., 1981).

Plasman and Vray (1993) found that nearly 30% of mouse RPC are lymphocytes; these would be expected in the non-adherent population, in addition to monocytes. Given that the reduction in the numbers of *ts13* IC was often greater in the non-adherent population than in the adherent, the replication of *ts13* may be restricted in the lymphocyte component of these cells, as well as in the monocytes. The replication of *ts13* in the MLN and spleen was even more restricted than in the RPC. The vast majority of lymph node cells are lymphocytes, of which T-cells alone comprise 74% (Fung-Leung et al., 1994). In the spleen, approximately 89% of mononuclear cells were found to be lymphocytes (Harrington et al., 1997). T lymphocytes made up 33% of the total mononuclear cells, although monocytes and macrophages contributed an additional 9% (Harrington et al., 1997). Given that both the spleen and the MLN contain large numbers of lymphocytes, and that both of these tissues supported substantial replication of the *wt* virus, it appears unlikely that the virus therein was produced solely by macrophages. Thus, lymphocytes in the spleen and MLN may also be important target cells for Pichinde virus. It follows that restricted replication of *ts13* in these cells may further contribute to the markedly reduced growth in the spleen and MLN. In future studies, it would be of great interest to assess the replication in lymphocytes by separating spleen and lymph node cells from infected mice into subpopulations consisting

of CD4⁺ T cells, CD8⁺ T cells, and B cells, prior to assaying for virus. This would elucidate whether the reduced growth of *ts13* in a specific type of lymphocyte is a factor contributing to the attenuated phenotype.

Several attempts were made to infect lymph node cells *in vitro*, since it was expected that the behaviour of *ts13* therein would be indicative of its capacity for growth in lymphocytes. Disappointingly, these cultures demonstrated poor viability, even when concanavalin A (conA)-conditioned medium was included in the culture medium at concentrations up to 40%. However, the concentrations of IL-2 in this conditioned medium had not been measured, so it was not known whether sufficient quantities were present. Nevertheless, it was not possible to study the growth of *ts13* in these cells, as the cultures did not even support replication of the *wt* virus. If lymphocytes are indeed targets of infection, they are more likely to permit viral replication when in an activated state. This condition is fulfilled in the MLN *in vivo*, since lymphocytes are participating in an antiviral immune response and are rapidly proliferating. In future *in vitro* studies, it would of use to simulate this activated state by pre-treating MLN cells with a mitogen, or anti-CD3, or anti-immunoglobulin (Ig) prior to infection in order to induce proliferation of particular lymphocyte populations, T cells or B cells, respectively. If selecting for T cells, it would also be necessary to culture cells in known quantities of IL-2. It would also be necessary to examine the replication of *ts13* in specific lymphocyte components of the MLN and the RPC, to determine whether specific cell types other than macrophages also limit the growth of *ts13*.

This communication highlights a possible difference in the biology of Pichinde virus and LCMV. Macrophage-tropic and lymphocyte-tropic variants of LCMV have

been isolated from the spleens and lymph nodes, respectively, of persistently-infected mice (King et al., 1990). Plaque-purified viruses displayed heightened growth in either macrophages or lymphocytes or both, and reached greater levels in the spleen and serum than did the *wt* virus. The magnitudes of these growth differences were several \log_{10} , which is on the order of those observed between *ts13* and *wtDA*. It is notable that the *wt* LCMV parental strain ordinarily does not possess a strong tropism for either cell type (King et al., 1990). Both types of LCMV variant were able to establish persistent infections in immunocompetent adult mice. In the case of Pichinde virus, even the *wt* requires macrophages in order to produce a successful infection. Impaired growth in these cells, and possibly in lymphocytes, apparently results in reduced growth *in vivo*, and is associated with a mutant phenotype. In the guinea pig model, increased growth of Pichinde virus variants in macrophages was associated with enhanced virulence, rather than a persistent infection. These findings may be indicative of more general differences in the behaviours of the New World and Old World viruses.

Early events, such as the binding to and entry into target cells, influence the success of a virus infection (Tyler and Fields, 1996). In the macrophages and RPC *in vitro*, the numbers of *ts13*-infected cells immediately following adsorption (0 hour time point) were consistently less than those of *wtDA*-infected cells. The extent of the reduction was similar for the two cell populations. Since the cell-associated virus detected at this time is believed to be virus which has bound, but not yet begun replication, these results suggest that the binding of *ts13* to target cells may be impaired relative to that of the *wt*. That this might be relevant *in vivo* was demonstrated by the markedly reduced numbers of IC in the RPC of *ts13*-infected mice at 3 hrs p.i.. This

reduction was noticeable in both RPC populations, but it was greater in the adherent population, although the significance of this is not known. Given that infectious virus detected 3 hrs p.i. is roughly indicative of the ability of Pichinde virus to adsorb to and enter target cells in the RPC, the reduced quantities of *ts13* suggest that these steps may be impaired, possibly even more so in the macrophage population. Since the RPC are the first cells encountered by the virus following an IP inoculation, the potentially reduced entry of *ts13* into these cells may compromise its initial replication. As a result, the levels of *ts13* in the RPC may not reach those of the *wt* virus, thereby limiting the spread to other sites, and resulting in reduced detection in other organs. In an analogous manner, *ts13* may undergo restricted initial replication in blood monocytes, which could partially account for the attenuated growth observed following IV inoculation.

In the murine embryonic fibroblasts, marked differences in the numbers of IC for the two viruses were observed at the 0 hr time point, reinforcing the suggestion that altered binding may be one explanation for the growth phenotype of *ts13*. It should be reiterated that this result may not necessarily reflect the binding of *ts13* to adult fibroblasts. If the attenuation of this virus does indeed result from an inability to replicate efficiently within murine cells, it would be analogous to that of the mouse-attenuated C167 strain of Junin virus (Scolaro et al., 1989). This Junin strain demonstrated restricted growth in murine embryonic fibroblasts, occurring, due in part to reduced binding. To confirm these theories regarding possible aberrant binding, it would be essential to quantify the relative binding abilities of *ts13* and *wtDA*, both to macrophages, and to murine fibroblasts in general, and to compare their velocity constants for the cell attachment reaction. This may be accomplished by several

methods, including measurement of the adsorption of radiolabeled virus to target cells (Nakhasi et al., 1989).

Although the cellular receptor for Pichinde virus is not known, a candidate receptor for LCMV was identified (Borrow and Oldstone, 1992). This putative receptor is a 120-140 kDa protein present in the membranes of susceptible cells. The protein is glycosylated, but carbohydrate moieties did not appear to play a role in virus binding. A wide range of cell lines is permissive for arenavirus infection. LCMV replicates in mouse (MC57, Balb C17), rat (Rin), hamster (BHK-21), monkey (Vero), or human (HeLa S3) cells, each expressing this putative receptor (Borrow and Oldstone, 1992). In contrast, the lymphocyte cell lines RMA (T-lymphocyte) and WIL-2 (B-lymphocyte) are not susceptible to virus infection, and, not surprisingly, these lack the potential receptor. Given that Pichinde virus is known to infect many of the above fibroblast cell lines (Dr. K. Wright, unpublished data; Young et al., 1986), it may also employ, *in vitro*, a receptor similar to that of LCMV. Pichinde virus was also shown to infect human differentiated monocytic cell lines (Polyak et al., 1995; Polyak et al., 1991; Lewis et al., 1989). It would be of interest to determine whether the receptor on these cells is the same as that on the fibroblast cells, and whether this is the receptor used *in vivo*. It should be noted that the differences in the levels of *ts13* and *wtDA* at the 0 hr time point are less marked than those at subsequent time points. If the quantities of the two viruses at this time are taken to approximate the relative binding abilities, it is thus possible that events subsequent to binding to and entry into host cells also play an important role. It must be noted that it will not be possible to quantify the contribution of impaired binding until appropriate binding assays are performed.

It was proposed above that initial events resulting in the limited replication in the RPC, possibly at the level of entry, compromised the spread of *ts13* from the site of inoculation, resulting in reduced detection in the other organs. Although the extent of the reduced replication observed in the RPC was sufficient to support this theory, it does not completely account for the markedly restricted replication in the spleen and MLN. The attenuated phenotype of *ts13* may actually result from restrictive influences acting at two stages, the first at the level of replication in RPC, and the second at the spread to or replication in the individual secondary tissues. Although it is uncertain whether this second factor is present, it may involve specific cell types in the target tissues. As mentioned, the spleen contains approximately 9% macrophages and 89% lymphocytes (Harrington et al., 1997), while MLN are highly enriched for lymphocytes. It is possible that the strikingly diminished quantities of *ts13* observed in comparison to the *wt* resulted from inefficient replication in one or both of these cell populations in the secondary tissues, in addition to reduced spread from the site of virus entry. To assess the possibility that secondary sites of replication are further restrictive, it would be necessary to isolate specific cell populations, such as macrophages and lymphocytes, from the spleen and lymph nodes, and to examine the replication of *ts13* in these cells *in vitro*. Alternatively, flow cytometry using double-staining techniques could be employed. This method would determine the particular cell types which harbour virus in the tissues of infected mice, by analysis of viral protein expression in cells with specific surface markers. Such analyses were not possible, since the available Pichinde virus-specific polyclonal serum reacted to mock-infected cells by flow assessment and no monoclonal antibody to viral surface proteins is available. However, it may be possible to stain

lymphocyte subsets, then permeabilize cells and stain for virus using monoclonal antibodies to the nucleoprotein. It would be important to apply these studies to macrophages derived from the various tissues, given that macrophages may differ widely in their phenotypes depending on their physiological location (Abbas et al., 1991). This would identify the macrophage populations which are most restrictive for *ts13* growth.

The characterization of an attenuated viral strain is further enhanced by genetic analyses. By identifying genetic changes which give rise to attenuated growth or virulence, it becomes possible to elucidate the mechanisms involved. Such studies are of particular importance in the development of vaccines, since the stability and therefore, safety, of the vaccine can be assessed on the basis of the number of known attenuating mutations (Snyder et al., 1988). An investigation of the responsible mutations in *ts13* was begun with the sequencing of the S segment genes and intergenic region. When the GP-C of *ts13* was cloned and sequenced (K. Wright, unpublished results), a single amino acid change, which converted an Asn residue to a Ser, was identified in the GP-1 portion of the gene. This mutation eliminated a potential site of N-linked glycosylation. The results presented here suggested that this potential site was utilized in *wTDA* and was missing in *ts13*. The pattern of glycosylation affects the conformation of viral envelope proteins, a factor which influences the efficiency with which a virus may bind to host cell receptors (Nakhasi et al., 1989). Altered glycosylation may change this 3-dimensional structure, potentially impairing the affinity of the envelope protein for the receptor, which may result in the reduced binding suggested by the growth experiments. A similar situation was documented for an attenuated vaccine strain of Rubella virus (Nakhasi et al., 1989). This mutant had lost a glycosylation site on an envelope protein, resulting in

faster migration on polyacrylamide gels, compared to the *wt*. Accordingly, this vaccine virus demonstrated reduced binding to target cells.

That a change in an outer envelope protein is potentially involved in the attenuation of *ts13* is not unexpected, given that, among the arenaviruses, such changes have exerted profound effects on virulence and on tropism. The attenuated phenotype of Junin strain C167 has been explained in part by reduced binding to murine cells, by virtue of sequence changes in its major glycoprotein (Scolaro et al., 1990; Scolaro et al., 1989). In addition, two amino acid mutations were identified in the GP-2 protein of the attenuated Candid#1 strain of Junin (Albarino et al., 1997). Although these did not have effects on glycosylation and on the cleavage site, they resulted in the loss of 4 β -turns, which produced a striking alteration in the predicted secondary structure of the protein. The tropism of LCMV can be strongly influenced by a single amino acid change in GP-1 (Ahmed et al., 1991). Variants of LCMV with tropisms for lymphoid tissue have an amino acid change at position 260 of GP-1 which correlates with the ability to persist in adult mice and the failure to elicit CTL responses (Ahmed et al., 1991). Common to all of these studies was the observation that only a small number of mutations, or even a single mutation, were adequate to produce dramatic changes in the growth phenotype.

For *ts13*, the effects of reduced glycosylation may also be manifest later in the virus replication cycle, possibly at the stage of GP-C cleavage, or transport of the glycoproteins to the surface of infected cells. Insufficient availability of GP-1 and GP-2 for incorporation into virions may diminish the quantities of infectious virus budding from cells, resulting in fewer *ts13*-infected cells within an organ or tissue. The importance of glycosylation to infectivity was shown by studies with Junin virus, and

with LCMV, in which inhibitors of certain key steps in glycosylation and transport substantially reduced the levels of budded virus (Damonte et al., 1994; Wright et al., 1990).

Since attenuation frequently arises from multiple mutations, the NP of *ts13* was also sequenced, and compared to that of *wtDA*. Two of the nucleotide differences between the viruses were silent, and were unlikely to be determinants of the attenuated phenotype. A third nucleotide change converted the Ile residue at amino acid 275 of *wtDA* to a Thr. This did not alter the overall charge of the protein, but did substitute a non-polar amino acid with a polar one. Notably, this created an additional site for phosphorylation on threonine residues. An endogenous nucleocapsid-associated kinase activity was previously reported for LCMV (Howard and Buchmeier, 1983). The major viral substrate for this kinase was NP, which became phosphorylated on Ser and Thr residues. These products were proposed to be involved in the regulation of replication and virus maturation, possibly by changing the conformation of NP in manner that altered its binding to the viral genome or that modulated the accessibility of the viral polymerase to the RNA template (Howard and Buchmeier, 1983). The increased potential phosphorylation of the NP of *ts13* could therefore modify the control of viral replication. If this alteration produces a negative effect on transcription and replication, it will further contribute to the attenuated phenotype.

In the Candid #1 vaccine strain, mutations in the nucleoprotein, in addition to those described in the glycoprotein, distinguished this attenuated virus from its more virulent ancestral viruses (Albarino et al., 1997). A single amino acid change in the amino-terminus altered the charge of the protein, relative to the virulent strains. NP has been proposed as the

arenaviral anti-terminator protein, permitting the establishment of genomic replication (Albarino et al., 1997; Auperin et al., 1984). Therefore, changes in this protein may affect its interaction with the viral RNA, and may disturb the regulation of replication.

As evidenced by the Sabin strains of poliovirus, mutations within non-coding regions have also been important determinants of attenuation, by their effects on RNA secondary structure (McGoldrick et al., 1995; Guillot et al., 1994). In the case of *ts13*, the intergenic region between the GPC and NP genes was found to contain a mutation within the putative stem-loop structure. The change in *ts13* results in a tighter secondary structure. This may further stabilize the stem-loop, and could hinder the transition from transcription to replication, which requires the passage of the viral polymerase past this region. Thus, the intergenic region of the S RNA of *ts13* also contains a potential attenuating mutation.

However, it must be mentioned that additional genetic changes may be encoded on the L RNA, which encodes both the polymerase, L, and the Z protein (Lukashevich et al., 1997; Djavani et al., 1997). In future studies, it would be particularly important to examine the L protein, given that, for many viruses, mutations in polymerases contribute to the attenuated phenotype (Skiadopoulos et al., 1998; Herlocher et al., 1996; Griot et al., 1993).

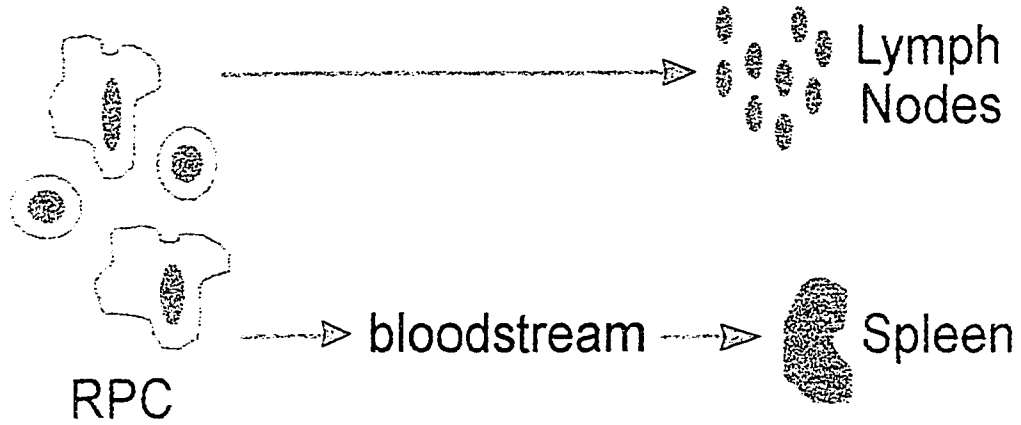
It is interesting to note that no genetic analyses of the virulent AdPIC strain of Pichinde compared to the attenuated prototype virus have ever been performed, in spite of the fact that this guinea pig model system has provided very promising groundwork for the study of arenavirus virulence and attenuation.

It is not known whether the aforementioned mutations in the S RNA segment of *ts13* also confer the *ts* phenotype. Given that the *ts* mutation had originally been mapped to this segment, it is likely that one or more of the changes identified in this work do play a role. Initial attempts to repeat the published experiments that demonstrated a cleavage defect in GPC, and suggested the presence of a *ts* mutation in this protein, were not successful. These additional experiments indicated that *ts13* showed a cleavage phenotype which was intermediate between the *wt* virus and another *ts* mutant with a clear reduction in GPC cleavage. This suggested that further work must be done to fully characterize the *ts* defect of *ts13*. The role of the above amino acid changes in *ts13* could be determined with greater certainty if methods to engineer mutations within the Pichinde genome, rescue these into infectious virus, and study the phenotypes of the progeny were available.

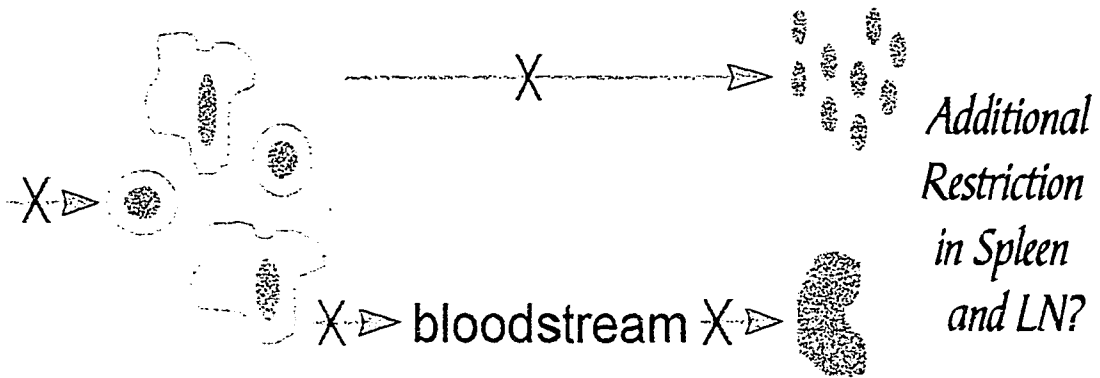
The complete basis for the attenuated phenotype of *ts13* is still not entirely known. Nevertheless, a partial mechanism can be proposed as a result of these studies. This is detailed in Figure 9. We propose that, in animals inoculated with the *wt* virus, an initial burst of viral replication occurs in cells near the site of inoculation, such as the RPC. The virus replicates to substantial levels and then spreads by entering the bloodstream and traveling to other organs, such as the spleen. The virus may spread to the lymph node via lymphatic or hematogenous spread. A secondary burst of replication then occurs at these sites, permitting the establishment of a widespread infection throughout the host. Compared to the *wt* virus, *ts13* exhibits poor initial replication in cells such as the RPC. This reduced growth may involve impaired entry into host cells, both monocytes and macrophages, and maybe lymphocytes, although later events such as

Figure 9. Proposed Mechanism for the Reduced Growth of ts13 In Vivo. *Top Panel:* Proposed course of initial replication and dissemination during an infection with the *wt* virus. *Bottom Panel:* Hypothetical restricted initial replication, dissemination, and secondary infection during an infection with *ts13*. Potential stages where restrictive factors may act are indicated by red symbols.

wtDA



ts13



transcription and replication may also be affected. This restriction may be more general and may extend to all murine cells. As a consequence, the burst of primary replication for *ts13* is suboptimal, thereby reducing the amount of virus available for spread to other tissues. Though this remains to be determined, there may also be a second stage of restriction, occurring at sites of secondary replication and potentially involving other cell types, possibly dendritic cells or lymphocytes. This would account for the extreme reduction in the levels of infectious *ts13* in the spleen and MLN, relative to *wtDA*. As an alternative, Pichinde virus replication may still be restricted to macrophages, but splenic macrophages may be more susceptible to infection with the *wt* virus than are macrophages and monocytes in the RPC, thus explaining the increased amounts of *wtDA* relative to those of *ts13*.

The observation of the attenuated phenotype of *ts13* was quite intriguing, given that no attenuated mutants of Pichinde virus have ever been described prior to this study. Since Pichinde virus poses no known risk to human health, it and its mutant *ts13* are safe for laboratory investigation. As described above, the properties of *ts13* were not dissimilar to those of other attenuated arenaviruses, notably strains of Junin virus, in terms of the *ts* phenotype, the reduced replication in an experimental animals, and the presence of potential attenuating mutations in S segment genes (Ceriatti et al., 1986; Contigiani et al., 1993; Laguens et al., 1983; Scolaro et al., 1990). Characterization of strains such as *ts13* provides valuable information which can be used to deduce common properties that are desirable in attenuated arenaviruses and the genetic changes required to produce these phenotypes. This, in turn, will become very useful in the design of

candidate live, attenuated vaccines, the need for which is increasingly urgent in the midst of new, emerging arenavirus pathogens.

In addition to decreased virulence, immunogenicity is an important requirement for an effective vaccine (Ellis, 1994). It will be necessary to determine whether an attenuated arenavirus such as *ts13* induces an immune response. Preliminary experiments suggest that *ts13*-infected mice do seroconvert (ELISA titre 1/100), although the titres of Pichinde-specific antibodies were lower than those of mice infected with *wtDA* (ELISA titre 1/1600), possibly reflecting the reduced replication of *ts13 in vivo*. To further complete the characterization of *ts13*, an examination of its behaviour in animal systems, such as neonatal mice (Wright et al., 1995) or MHA hamsters (Murphy et al., 1977), in which mortality is the usual outcome of Pichinde virus infection, will be required. This will determine whether *ts13* is attenuated for virulence, as well as growth. If *ts13* does display reduced virulence, its ability to protect animal subjects against a challenge with the *wt* virus must be assessed, an investigation which could have important implications in the future development of vaccines.

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APPENDIX A: PRIMERS

Primer	Region of Genome	Sense	Sequence
3' S-RNA	nt 3419-3386	viral complementary	5'-CGCACAGTGGATCCTAGGCG ACACTAGATCACGC-3'
P-S-2	nt 1370-1388	viral	5'-CCCCTTTGGCACTGACAGA-3'
cNP-2	nt 2874-2856	viral complementary	5'-GGTGTGTG AAGGTCTGGG-3'
cNP-3	nt 2332-2313	viral complementary	5'-CTCAAAGCCAAGTGCTATCC-3'
cNP-4	nt 1792-1772	viral complementary	5'-CACTGCAAACCCCCACTGTGC-3'
vNP-5	nt 1901-1920	viral	5'-GCACTGAGCTTCACGTCAAG-3'
vNP-6	nt 2444-2462	viral	5'-GGGTTTCCTTTGCCCGGGTG-3'
vNP-7	nt 2878-2996	viral	5'-GGCTGAGACAGGCCTTCGG-3'

APPENDIX B: CONCENTRATIONS OF CELLS ASSAYED FOR INFECTIOUS VIRUS

Cell Suspension*	Range of Cell Concentrations per ml
RPC, <i>in vivo</i>	1.22 x 10 ⁵ - 1.15 x 10 ⁷
MLN, <i>in vivo</i>	4.82 x 10 ⁶ - 5.41 x 10 ⁷
RPC, <i>in vitro</i>	1.00 x 10 ⁵ - 5.00 x 10 ⁶
J744A.1	5.00 x 10 ⁶
MEF	1.00 x 10 ⁶

* Types of cell suspensions used for the infectious centers assays were: resident peritoneal cells (RPC) or mesenteric lymph node cells (MLN) from infected mice; RPC, infected *in vitro*; J744A.1 macrophage cell line, infected *in vitro*; primary murine embryonic fibroblasts, infected *in vitro*. Details regarding the *in vitro* infections can be found in Materials and Methods and in Results.

CURRICULUM VITAE

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Synopsis

A dependable, hard-working, and flexible laboratory technician/graduate student, with excellent communication skills.

Academics

- 1995-present Master of Science, Microbiology and Immunology, University of Ottawa. In progress: expected summer 1998.
Thesis Title: A Mouse-Attenuated Temperature Sensitive Mutant of Pichinde Virus: *In Vivo* and *In Vitro* Characterization.
Thesis Supervisor: Dr. K. Wright.
- 1991-1995 Bachelor of Science (Honours), Microbiology and Immunology, McGill University.
Dean's Honour List 1992-1995.
- 1997 Ontario Graduate Scholarship.
- Spring 1996 Sam Sadava Memorial Scholarship.
- 1991-1995 Canada Scholarship Award.
- 1992-1993 J.W. McConnell Award (McGill).
- 1991-1992 J.W. McConnell Entrance Award (McGill).

Employment Experience

- Present Laboratory technician. Diabetes Research, Health Canada.
Tasks: Planning and participating in animal studies involving the effect of diet on diabetes incidence; laboratory management and maintenance.
Supervisor: Dr. F. Scott.
- 1997-1998 Laboratory technician. Hepatitis Laboratory, Health Canada.
Tasks: PCR-based diagnosis of Hepatitis B virus.
Supervisor: Dr. R.K. Chaudhary.
- 1997 Project support (part time).

- Ottawa Regional Cancer Centre.
 Tasks: Studies of the susceptibility of PKR-deficient cell lines to virus infection.
 Supervisor: Dr. J. Bell.
- 1995-1996 Demonstrator for the Medical Microbiology course offered to medical students each fall by the Faculty of Medicine of the University of Ottawa.
 Supervisor: Nancy Delcellier.
- Summer 1995 NSERC funded Research Assistant. Department of Microbiology and Immunology, University of Ottawa.
 Tasks: Viral gene expression studies in tissue culture.
 Supervisor: Dr. K.E. Wright.
- Summer 1994 NSERC funded Research Assistant. Department of Microbiology and Immunology, University of Ottawa.
 Tasks: Cloning of viral genes.
 Supervisor: Dr. K.E. Wright.
- Summer 1993 NSERC funded Research Assistant.
 Department of Microbiology and Immunology,
 University of Ottawa.
 Tasks: Construction of recombinant vaccinia virus.
 Supervisor: Dr. K.E. Wright.
- Winter 1992 Volunteer at Montreal Neurological Hospital.
- Summer 1992 Assistant Maid, Molly Maid domestic services.
- 1990-present Occasional work with Kanata Faces ticket sales and face-painting.

Laboratory Skills

- Animal Experimentation:
 Virus inoculation of laboratory mice; harvesting of tissues such as: spleens, livers, kidneys, lymph nodes.
- Cell Culture: Culture of mammalian cell lines, and cells explanted from mice.
- Immunological Techniques:
 Immunofluorescence microscopy; flow cytometry; ELISA.
- Molecular Biology:
 PCR; extraction and purification of nucleic acids; cloning; electrophoresis; plasmid-mediated transfection of mammalian cells; radioimmune precipitation of proteins.
- Virology: Cultivation, quantification, purification of animal viruses.

Experience with viruses such as: Pichinde, LCMV, Vaccinia, Mumps, Influenza, Reovirus, Hepatitis B and C.

Additional Skills

Languages: Excellent oral and written skills in English.
Competent oral and written skills in French.

Computers: Familiarity with DOS and Windows applications, including Word, WordPerfect, Quattro Pro, Lotus 1-2-3, Sigma Plot, FigP, and Powerpoint.

Interpersonal Skills:

Attended two day workshop for demonstrators given by the University of Ottawa (September 1996). Topics included sensitivity to students of diverse cultural backgrounds and techniques for leading effective tutorials.

Taught flute and tutored mathematics (1988-1990.)

Activities

Present Member of Ottawa Flute Association flute choir.

1995-1997 Member of Kanata Symphony Orchestra.

1992-1995 Member of CAMMAC Orchestra.

1991 Royal Conservatory of Music grade 9 practical flute.

1993 First Aid/CPR, St. John Ambulance.

Posters, Presentations and Publications

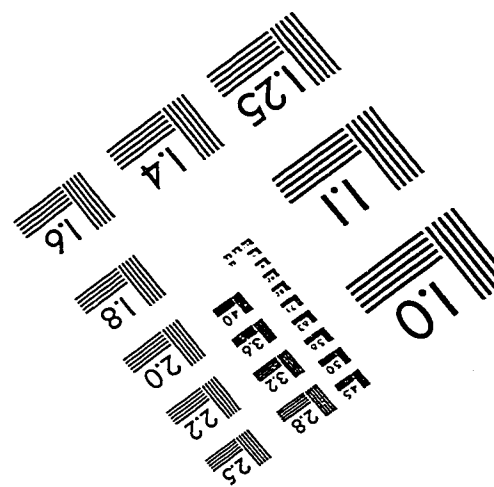
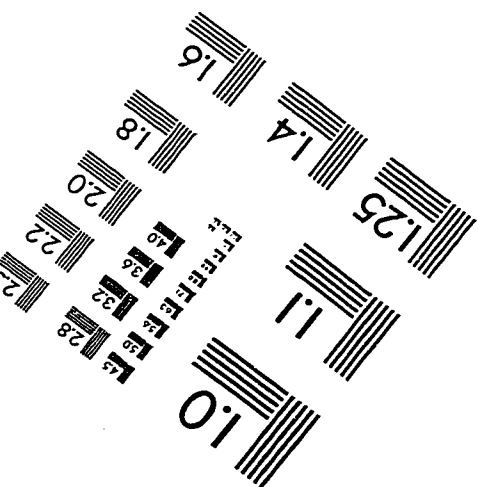
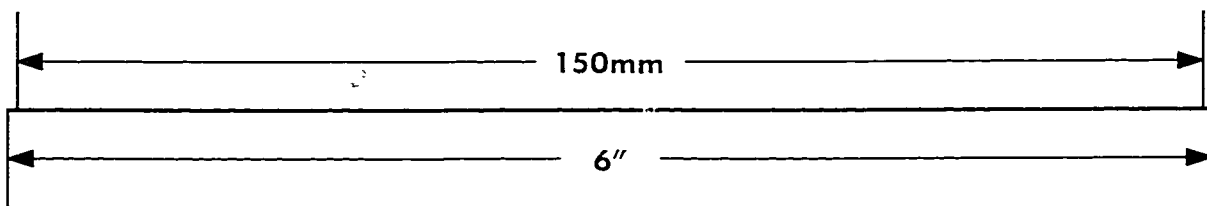
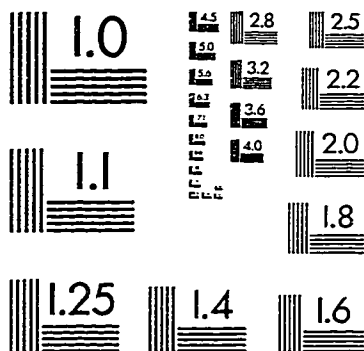
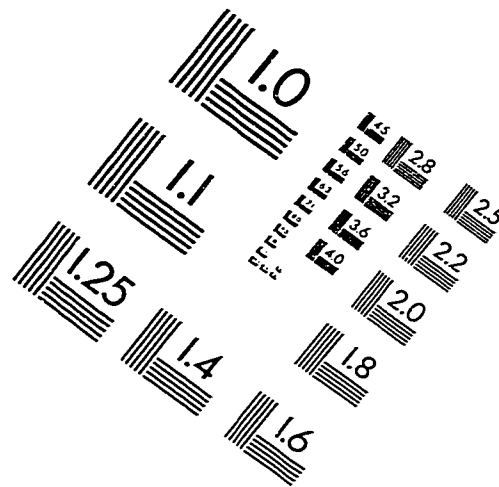
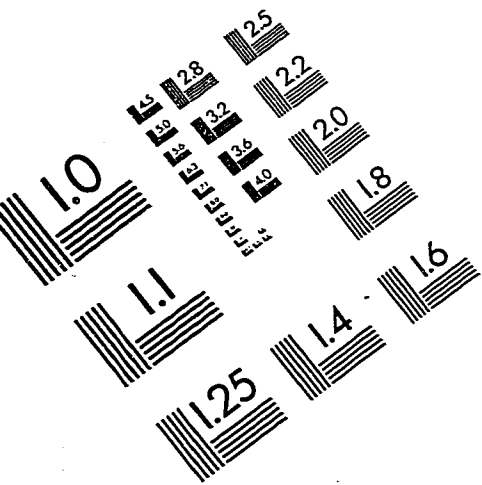
Poster presented by K.E. Wright at the Annual Meeting of the American Society of Virology (ASV), 1995, in Austin, Texas: "Amino Acid Changes in the Major Glycoprotein (GP-1) of Pichinde Virus Important for Cleavage of Precursor Glycoprotein." (K.E. Wright, A. Bergeron, S. Xhu, and H. Gruber)

Talk presented by H. Gruber at the 1997 Meeting of The Canadian Society for Microbiologists (CSM), June 16th, 1997, in Quebec City, Quebec: "A Temperature Sensitive Mutant of Pichinde Virus is Attenuated in Mice." (H. Gruber, and K.E. Wright)

Talk presented by H. Gruber at the 1996 Annual Meeting of The American Society for Virology (ASV), July 14th, 1996, in London, Ontario: "A Temperature Sensitive Mutant of Pichinde Virus is Attenuated in Mice." (H. Gruber, and K.E. Wright)

Paper submitted for publication: "A Temperature Sensitive Mutant of Pichinde virus is attenuated in adult mice, and shows reduced growth in macrophages."

IMAGE EVALUATION TEST TARGET (QA-3)



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