Title
Correlation of the pathologic potential of Theiler's Murine Encephalomyelitis Virus (WW strain)

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Correlation of the Pathologic Potential of Theiler's Murine Encephalomyelitis Virus (WW Strain) With Virion Polypeptide Composition

by

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DISSERTATION

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"Those who have handled sciences have either been men of experiment or men of dogmas. The men of experiment are like the ant; they only collect and use; the reasoners resemble spiders, who make cob webs out of their own substance. But the bee takes a middle course; it gathers its material from the flowers of the garden and of the field, but transforms and digests it by a power of its own..."

Francis Bacon

NOVUM ORGANUM,
Book I, XCV

DEDICATION

I dedicate this work to my parents, John and Connie Stroop; to my father who instilled in me the need to be a truth seeker, and the usefulness of experimentation as a tool to separate fact from legend, and to my mother for her compassion, understanding, and tolerance as I pursued many projects guided more by curiosity than by wisdom.

But most importantly, I dedicate this work, as I have my life, to my wife, Brandy, whose understanding and encouragement gave me the strength to continue, whose tenderness and compassion provided me with tranquility in sometimes otherwise stormy seas, and whose unselfish gift of love has made it worthwhile.

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I wish to extend my thanks and acknowledge my advisor, Dick Baringer, for his professional and personal support, and for the freedom he extended to me to pursue all avenues of investigation. I also wish to thank Jerry Wolinsky for his encouragement to undertake these studies and for the many insights he provided into the methodology and design of studies dealing with viral pathogenesis. I would also like to thank Ken Johnson for his unselfish and generous gifts of time and worthwhile discussions.

I wish to acknowledge the members of the Neurology Research Laboratory, past and present, including Toni, Peggy, Paula, Bill, Carol, Jinny, Roberta, Laurie, June, Chris and Hill, for their timely suggestions and for making the work environment so pleasant. I would also like to thank Richard, Don, Barbara, Nick and Denise for their friendship, caring, and understanding of the pursuit of the Ph.D. I thank Bob and Joyce McKendall for their friendhip, and providing a touchstone with the outside world. I especially wish to express my gratitude to Bob for his unending role as the devil's advocate, and for constantly raising issues I oftentimes would have preferred to have left alone.

My special thanks to Judy Rohrer, Prima Conde and Nancy Torelli, not only for their expert typing and editorial advice, but also, and perhaps most importantly, for their

patience and understanding throughout the long and arduous process of preparing this work.

I would also like to thank the members of my Dissertation Committee, in addition to my Chairman and Advisor, Dick Baringer, Drs. Joe Caldwell, Leon Levintow, Bob Stern, and Ed Smuckler, for their support and interest, not only in my scientific pursuits, but also in my career aspirations.

THE PROBLEM

I see cells and viruses interacting with each other,
the complexities are overwhelming, I feel I'll smother.
The more I read and think about everything,
the less I really know about anything.

Learning about disease is no easy feat,
coupling virology with pathology is just but the least.
Immunology bounded with the others in a pathogenic pact,
with its inherent half-truths and theories short on fact.

A thread of truth I envision must lie through it all,
a unifying concept of this pathogenic free-for-all.
For it seems that when viruses are the cause of disease,
they evoke host responses as varied as you please.

Unraveling these complexities is the goal I seek,
to make sense out of the interactions, each which seems unique.

wgs

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ABSTRACT

Theiler's Murine Encephalomyelitis Viruses (TMEV) comprise a group of murine picornaviruses. A biphasic disease was produced in weanling mice following intracranial (IC) inoculation of the WW strain of TMEV (TMEV(WW)). The first disease phase was an acute polioencephalomyelitis accompanied by hind-limb paralysis, and appeared histologically within 2-7 days post-inoculation. Mice which survived the acute phase developed a characteristic hind-limb gait disturbance 20-30 days later. This second phase of disease was characterized histologically by chronic progressive demyelinating lesions primarily of the lateral columns of the spinal cord. Cell-free virus could be recovered from central nervous system tissues throughout the late disease.

TMEV(WW) was found to be unable to produce direct cytopathic effect (CPE) when inoculated onto cell cultures as a brain homogenate or when infected brains were cocultivated with a variety of cell types. The virus was adapted to produce CPE in tissue culture following blind-subpassage. Tissue culture adapted TMEV(WW) (TC-TMEV(WW)) was found to be unable to produce acute polioencephalomyelitis following IC inoculation of mice, but retained a demyelinogenic potential.

To determine what caused the apparent attenutation of TMEV(WW) during tissue culture adaptation, purified preparations of TC-TMEV(WW) and brain-derived virus (B-TMEV(WW))

were subjected to biophysical and biochemical comparative analyses. Both viruses were found to have biophysical characteristics in common with other picornaviruses. They were 26 nm particles, had estimated sedimentation coefficients of 156 S ($S_{20}\omega$), and bouyant densities of 1.34-1.35 gm/cm^3 in CsCl. Both TC- and B-TMEV(WW) contained picornaviral capsid proteins VPO, VP1, VP2, VP3 and VP4 with molecular weights of 40, 37, 34, 27 and 6 kilodaltons, respectively. B-TMEV(WW) contained two additional viral specific polypeptides with molecular weights of 58,000 and 64,000 daltons, and were designated P58 and P64, respectively. Both P58 and P64 had surface oriented tyrosine residues in intact virions. P58 was found to be an apparent disulfide-linked precursor of VP2 and VP3 which could be reduced in vitro. Interestingly, TC-TMEV (WW) lacked P58.

Because B- and TC-TMEV(WW) appeared to differ biochemically in that only B-TMEV(WW) contained P58, and biologically in their pathogenic potential, the presence of P58 appeared to correlate with an ability of B-TMEV(WW) to induce polioencephalomyelitis. This postulate was tested by assessing the encephalitogenicity of B-TMEV(WW) pretreated with a reducing agent in vitro to partially eliminate P58 bearing virions from the inoculum. Treated virus preparations produced a significant delay in the onset of acute polioencephalomyelitis, measured by clinical signs, and death, compared to identical preparations of untreated virus. When identical experiments were performed on TC-

TMEV(WW), no change in infectivity was observed following treatment of the virus with the reducing agent.

These results indicated that multiple variants of TMEV(WW) extisted in acutely infected mouse brain. Loss of one variant, identifiable by the capsid precursor protein P58, apparently as a consequence of tissue culture adaptation, produced a viral population devoid of encephalitogenic potential. Partial reduction of P58 bearing variants, by chemical treatment in vitro, caused a decrease in encephalitogenicity. Suggested by these studies is the possibility that the process of tissue culture adaptation of B-TMEV(WW) selected against an encephalitogenic variant (bearing P58), and raises the possibility that the late demyelinating disease produced in mice may be related to the other variant (bearing P64). Possible mechanisms of Theiler's virus persistence and its relationship to the biphasic disease produced in mice are discussed.

INTRODUCTION

Historical Perspective:

In 1933 Max Theiler reported the observation of an animal which had spontaneously developed hind limb paralysis in his mouse colony (323). Theiler was able to isolate an infectious agent from the paralyzed mouse by serially passaging a suspension of central nervous system tissue homogenate from mouse to mouse by intracerebral inoculation (323). Inoculated mice developed hind limb paralysis clinically identical to the spontaneously paralyzed mouse initially observed (324,326). The infectious agent was shown to be approximately the same size as poliovirus as determined by collodian membrane filtration (326). Because of the small size of the filterable infectious agent, Theiler correctly concluded that the agent was a virus and was responsible for paralysis seen as the clinical manifestation of infection.

Theiler isolated several strains of this spontaneous mouse encephalitis virus during the early 1930s and characterized some of their physical properties and the pathogenesis of viral infection (326,327). The original strains of Theiler's virus have become known as the TO strains (Theiler's Original). The original sources of these TO strains are illustrated in Table 1. During the

TABLE 1

ORIGINAL SOURCES OF THEILER'S VIRUSES

STRAIN	SOURCE	REFERENCE
TO[a]	Recovered by Max Theiler from the central nervous system (CNS) of spontaneously paralyzed mice in the Rockefeller mouse colony; 1933-1937	323, 324
-I -II -V -4727		
-II	Given to Max Theiler by W. Sawyer. Mouse was previously inoculated with material containing human poliovirus.	323
-IV	CNS of a mouse inoculated with material containing human poliovirus.	323, 324
GDVII	CNS of a mouse inoculated 11 days earlier with material suspected of containing yellow fever virus; 1940.	326
FA	CNS of a mouse inoculated with the 525th passage of the French Strain of Yellow Fever virus. Mouse had spasmodic movements, was hyperexcitable and had convulsions. Paralysis was rare in mice which received subpassages of this strain; 1940.	326, 327
TO-intestinal	Intestinal contents and intestinal mucosa of normal mice and mice experimentally inoculated with Theiler's virus strains; 1940.	231
DA	CNS of a spontaneously paraylzed mouse in the Harvard mouse colony; 1940.	63
WW	CNS of a mouse (ICR Strain) which received intracranial and intraperitoneal inoculations of a clarified suspension of a multiple sclerosis plaque; 1977. Virus was subpassaged by intracranial inoculations of suckling mice.	348

[a]TO stands for Theiler's Original isolates (TO- I-V, -4727). Some strains not listed here have been derived from these TO strains.

course of work involving yellow fever virus Theiler and Gard isolated two additional strains of Theiler's viruses, the GD VII and FA strains which were shown by these investigators and others to be more neurovirulent and to have a higher degree of neuroinvasiveness than the TO strains following peripheral inoculation into weanling mice (326,327) (Table 1). Tables 2 and 3 tabulate the pathogenesis of the various strains of Theiler's viruses isolated to date following peripheral and intracerebral inoculation of weanling mice.

The announcement that Theiler had isolated and identified several strains of the virus which could produce encephalomyelitis in mice which looked clinically identical to poliomyelitis in humans and experimental monkeys caused a great stir of excitement among those scientists of the day investigating the pathogenesis of human poliomyelitis. Interest in the study of human poliomyelitis had increased dramatically since 1900 due to the increase in number of epidemics of poliomyelitis which had been observed on a global scale. The first reported epidemic of paralytic poliomyelitis was observed in Sweden (83) and the first reported case of the disease in the United States was in Vermont in 1894. The first epidemic of paralytic poliomyelitis was observed in New York in

TABLE 2

CHARACTERISTICS OF THEILER'S MURINE ENCEPHALOMYELITIS VIRUS INFECTION IN SUCKLING MICE FOLLOWING PERIPHERAL INOCULATION OF INFECTED CENTRAL NERVOUS SYSTEM (CNS) HOMOGENATES[a]

STRAIN	VIRULENCE[b]	NEURO-INVASIVENESS	CLINICAL SIGNS	HISTOPATHOLOGY OF DISEASE(S) PRODUCED
TO	very low[c]	low-none[c]	paralysis[c]	polioencephalo-myelitis[c] myositis[f]
TO-intestinal	very low[e,h]	low-none[e,h]	encephal-itis[e,f,i]	none[i,g]
DA	none[d]	low-none[d]	none[d]	myositis[d]
WW	none	none	none[m]	NR[p]
GD VII	high[j,h,k]	high[j]	paralysis[j] encepalitis[j,n]	meningoencephalitis[n] polioencephalitis[j] myositis[f]
FA	high[j,k,h]	high[j]	spasticity[j] convulsions[j] hyperreflexia[j]	polioencephalitis[j] myositis[f]

(CONTINUED)

TABLE 2 (CONT'D)

CHARACTERISTICS OF THEILER'S MURINE ENCEPHALOMYELITIS VIRUS INFECTION IN SUCKLING MICE FOLLOWING PERIPHERAL INOCULATION OF INFECTED CENTRAL NERVOUS SYSTEM (CNS) HOMOGENATES[a]

STRAIN	CNS PERSISTENCE	PRESENCE OF VIRUS IN OTHER ORGANS	VIREMIA	PRODUCTION OF IMMUNITY[l]
TO	NR	none[c]	no[e,j,h]	yes[c,j,h]
TO-intestinal	NR	NR	NR	yes[e]
DA	NR	NR	NR	yes[d]
WW	NR	NR	NR	NR
GD VII	NR	viscera[j,n]	yes[n]	yes[j]
FA	NR	viscera[j,n] nasal mucosa[j,o]	NR	yes[j]

[a]Table summarizes data obtained after intraperitoneal, subcutaneous, intravenous, intranasal and intramuscular injections and gastric gavage of infected mouse tissues

[b]Relative virulence determined by comparing the viral doses of strains required to produce mortality. Highly virulent strains require small doses to produce the same percent mortality produced by large doses of less virulent strains.

[c]Ref. 324

[d]Ref. 63

[e]Ref. 231

[f]Ref. 284

[g]Ref. 231

[h]Ref. 327

[i]Oltisky (231) reported that 20% of mice receiving feces filtrates intranasally developed experimental encephalomyelitis, but did not report histologic changes in CNS.

[k]Ref. 326

[l]Measured by resistance of mice to subsequent intracranial challenge or by tissue culture neutralizing antibody assays.

[m]Results reported in this study

[n]Ref. 177

[o]Virus detected in nasal mucosa only, following intranasal inoculation.

[p]Not Reported

TABLE 3

CHARACTERISTICS OF THEILER'S MURINE ENCEPHALOMYELITIS VIRUS INFECTION IN WEANLING MICE FOLLOWING INTRACRANIAL (IC) INOCULATION OF INFECTED CENTRAL NERVOUS SYSTEM (CNS) HOMOGENATES

STRAIN	VIRULENCE[a]	CLINICAL SIGNS	HISTOPATHOLOGY OF DISEASE(S) PRODUCED
TO	low[c,e,g]	paralysis[c,g]	poliomyelitis[c,f] polioencephalitis[c,i] myositis[d,e]
TO-intestinal	low[g,i]	paralysis[g]	poliomyelitis[g] polioencephalitis[g]
DA	moderate[e,l]	paralysis[e,d] chronic spasticity[p]	poliomyelitis[e,p] polioencephalitis[e,p] myositis[e] chronic demyelination[p,r,s,t,u]
WW	moderate[m,n]	paralysis[k] encephalitis[k] chronic spasticity[k]	polioencepahlitis[n] poliomyelitis[n] chronic demyelination[n]
GD VII	high[h,l]	encephalitis[d,i,o]	meningoencephalitis[h,e] polioencephalitis[h,e] myositis[e]
FA	high[h,l]	encephalitis[i]	meningoencephalitis[h,e] myositis[e]

(CONTINUED)

TABLE 3 (CONT'D)

CHARACTERISTICS OF THEILER'S MURINE ENCEPHALOMYELITIS VIRUS INFECTION IN WEANLING MICE FOLLOWING INTRACRANIAL (IC) INOCULATION OF INFECTED CENTRAL NERVOUS SYSTEM (CNS) HOMOGENATES

STRAIN	CNS PERSISTENCE	PRESENCE OF VIRUS IN OTHER ORGANS	VIREMIA	PRODUCTION OF IMMUNITY[b]
TO	yes[c] (48-163 dpi)[v]	NR[w]	no[c,f]	yes[h]
TO-intestinal	NR[j]	mesenteric glands and intestinal mucosa[g]	NR	yes[g]
DA	yes[e,p] (90 dpi)	NR	no[f,p]	yes[p]
WW	yes[n] (186 dpi)	NR	NR	NR
GD VII	NR[q]	viscera[h]	NR[f]	yes[h]
FA	NR[q]	viscera[h]	NR[f]	yes[h]

[a] Relative virulence determined by comparing viral doses to produce mortality (see legend to Fig. 2).

[b] Measured by resistance to subsequent IC challenge or by tissue culture neutralizing antibody assays.

[c] Ref. 324

[d] Ref. 284

[e] Ref. 63

[f] Daniels, et. al. (63) reported a transient viremia in suckling mice.

[g] Ref. 231

[h] Ref. 326

[i] Ref. 327

[j] Persistence in CNS not reported, but persistence in gut was reported (231, 325).

[k] Ref. 348

[l] Ref. 170

[m] Ref. 175

[n] Results reported in this study.

[o] Ref. 177

[p] Ref. 167

[q] Not reported with brain-derived stocks, but do persist with tissue culture derived stocks (168, 170).

[r] Ref. 175

[s] Ref. 174

[t] Ref. 172

[u] Ref. 60

[v] dpi, days post-inoculation

[w] Not Reported

1919 where approximately 9000 people contracted the disease (241).

Parayltic poliomyelitis is believed to have been a disease of man since ancient times. One of the first recognized illustrations of a human being showing a classic residual atrophied limb was the Stele for Ruma, a plaque in the collection of the Ny Carlsberg Glyptotek in Copenhagen showing Ruma offering to the Syrian goddess Astarte. It dates from the thirteenth century before Christ and was made during the reign of Pharao Ramses II. It is considered the earliest known recording of the effects of poliomyelitis. However, epidemics of paralytic poliomyelitis were not observed until relatively modern times and it was because of these clinically recognizable epidemics that the cause and study of paralytic poliomyelitis became the focus of world-wide, intense investigation.

Even before the cause of paralytic poliomyelitis had been discovered, and before epidemics of the disease had occured, the concept that paralysis was due to damage of the anterior horn cells of the spinal cord had already been put forth by Charcot in 1870 (44). Confirmatory evidence to support this concept was slow in coming. In 1888 Rissler presented evidence that the primary pathologic process leading to paralysis occurred

in nerve cells and that inflammatory changes were secondary (267). In 1909 Landsteiner and Popper (241) discovered that a virus was the cause of paralytic poliomyelitis. These investigators successfully transmitted the disease by passage of the virus into primates (monkeys). These monkeys developed clinical signs of paralytic disease. With development of an animal model of human poliomyelitis, it became possible to sequentially study the histopathological changes occurring in spinal cord following experimental inoculation.

In 1929, Hurst (126) and Bodian and Howe (25) in 1941 presented experimental evidence which indicated that neuron changes could occur independently of inflammatory changes during the preparalytic period of the disease. In a series of elegant studies, Bodian described the cytopathologic changes in motor nerve cells following infection with poliovirus (22,24). Bodian divided the stages of poliomyelitis in Rhesus monkeys into five stages: (i) the incubation stage which includes the latent and early preparalytic periods; (ii) the acute stage which includes the late preparalytic period and the period of increasing paralysis; (iii) the subacute stage or early recovery period; (iv) the convalescent stage or the period of continued recovery; and (v) the chronic stage.

Following experimental inoculation of poliovirus, the first microscopic change noted in motor neurons was diffuse chromatolysis of the Nissl substance. This occured during the preparalytic period. In the late preparalytic period, complete dissolution of the Nissl substance occurred. During the acute stage, neurons underwent more severe chromatolysis of the Nissl substance and some neurons developed vacoules in and dissolution of the cytoplasm. Only when severe cytoplasmic chromatolysis had occurred, were nuclear changes observed, which consisted of formation of acidophilic inclusion bodies. Karyorrhexis and pyknosis of nuclei were noted to occur only in those neurons showing very severe antecedent cytoplasmic injury.

Interestingly, changes in nerve cells of mild or severe degree during the preparalytic period were independent of inflammatory changes and sometimes occurred in regions where no inflammatory response was observed (22,126). Leukocytes were observed in considerable numbers in areas where nerve cell changes were so slight that they were not considered to be abnormal. The inflammatory response usually consisted of a very transient perivascular infiltrate of granulocytes followed by infiltration of mononuclear cells. The inflammatory response rose

during the acute stage, continued through the subacute stage and began to decline during the convalescent stage. During the latter two stages, the most numerous cell of the inflammatory response was the macrophage. These cells characteristically surrounded neurons forming nodules of neurophagia. During the convalescent and chronic stages some recovery of motor function occured. This recovery correlated histologically with the return to normal of the Nissl substance in those neurons which did not undergo severe chromatolysis or neuronophagia. The ultimate amount of recovery from paralysis appeared to depend on the number of motor neurons capable of repair following injury due to viral infection. The more neurons which innervated an affected muscle group and were capable of undergoing repair, the more complete was the recovery from paralysis.

During the epidemics of paralytic poliomyelitis, it became clear that infection with poliovirus could result in three types of disease differing in severity. The vast majority of cases of infection of poliovirus resulted in subclinical infection. Of those infections that become clinically manifest at all, most took the form of a minor illness characterized by fever, malaise, sore throat, headache, and vomiting. In one percent

or less of such cases, muscle pain and stiffness developed about three days later and were followed by classic paralysis which rapidly developed to its maximal extent. After a variable length of paralysis some degree of recovery of motor function could occur over the next few months but the paralysis which remained at the end of that time is permanent.

Theiler's virus infection of mice offered the first animal model of poliomyelitis by a virus in its natural host. Previous work on poliomyelitis had been performed by experimental inoculation of Rhesus monkeys (241,325). Experiments with monkeys had very serious disadvantages; first, experiments were expensive, thus limiting the amount of work possible; secondly, it had not been established that the disease produced experimentally in monkeys was the exact counterpart of the infection observed in man which is the only known natural host for poliovirus (83). In his early pathogenic studies, Theiler demonstrated the usefulness of the Theiler's virus mouse model as a model for poliomyelitis in humans.

Upon intracranial inoculation of Theiler's virus into weanling mice there followed a somewhat variable incubation period lasting from a few days to a few

weeks (324). At the end of the incubation period, animals presented with signs of hind limb paralysis. Depending on the strain of virus used, there were often few other signs (324). Histologically, the central nervous system tissue revealed the cellular damage characteristic of experimental poliomyelitis in monkeys mentioned above (22,266,269). Primary injury in paralytic Theiler's virus infections and in poliomyelitis occurred in the anterior horn cells of the spinal cord and was the cause of motor paralysis. Because of the similar size of poliovirus and Theiler's virus and the similarity between the histopathologies, Theiler characterized some of the physical properties of Theiler's virus and compared them with poliovirus. Table 4 summarizes the physical properties of Theiler's viruses.

It was clear that the Theiler's viruses and poliomyelitis shared many common physical properties (compare Tables 4 and 6) and caused apparently identical diseases in their natural hosts. Thus, to further establish the usefulness of Theiler's virus infection of mice as an animal model for the study of the pathogenesis of poliomyelitis in humans, Theiler and others sought further correlates between Theiler's virus infection of mice and natural or experimental poliovirus infection

in humans or monkeys. One of the striking features of natural poliovirus infection in humans is its age dependence. Paralytic poliomyelitis is a disease chiefly of children. Hence, its formerly common name, "infantile paralysis." It is useful in this regard to examine the epidemiology of paralytic poliomyelitis in that it sheds light on the age dependence susceptibility of infection and the cause of the world-wide epidemics during the first half of this century.

It is now widely appreciated that poliovirus is spread through a population chiefly by the fecal-oral route. Explosive epidemics have been known to result from contamination of water supplies by sewage. Underdeveloped countries, where community and personal hygiene are of a lower standard than they are in developed countries, allow the spread of virus from individual to individual within the community. For reasons that are not particularly clear, poliovirus infection in very young children is rather asymptomatic. Infection of a very young individual confers a life-long immunity to the type of poliovirus with which the individual was infected. Therefore, within a population of individuals in a relatively underdeveloped community, many of the adolescents and

TABLE 4

PHYSICAL AND BIOLOGICAL PROPERTIES OF THEILER'S VIRUS

I. PHYSICAL PROPERTIES									REFERENCES
A. pH Stability[a]: Stable at pH 3-8									305
B. Effect of Solvents:									
1. Stable in ether									325
2. Stable in fluorocarbon									89, 312
3. Destroyed in H_2O_2									325
C. Effect of Dessication:									
1. Loses 99.6% of infectivity after dessication and storage at -16° C.									326
D. Effect of Temperature:									
1. Destroyed after exposure to 50° C for 30 min.									326
E. Size: 1. 26-28 nm									176, 260, 312
F. Nucleic Acid: RNA									89
G. Density in Cesium Solutions:									
1.34 - 1.35 gm/cm^3									176, 312
H. Sedimentation Coefficient:									
156 S (S_{20^ω})									312
I. Polypeptide Composition:									
Tissue Culture Strains	Polypeptide Mol Wt X 10^3 daltons								
			VP0	VP1	VP2	VP3	VP4		
DA			36	34	32	25	6		176
GD VII			36	34	31	25	6		176
WW	P64		40	37	34	27	6		Results Reported here
Brain Derived Strains									
WW	P64	P58	40	37	34	27	6		312

(CONTINUED)

TABLE 4 (CONT'D)

PHYSICAL AND BIOLOGICAL PROPERTIES OF THEILER'S VIRUSES

II. BIOLOGIC PROPERTIES	REFERENCES
A. Serology:	
1. Serologically related to each other as determined by cross-neutralization tests in vitro and in vivo	169, 231, 327
2. Not related to poliovirus, yellow fever virus or cardioviruses by cross-neutralization tests in vitro or in vivo.	169, 325, 326
3. Related to cardioviruses and Vilyusik virus by complement fixation tests.	42, 146
B. Host Range:	
1. TO strains will grow only in mice.	231, 324, 325
2. FA and GD VII strains can be adapted to grow in cotton rats.	325
3. DA, FA, and GD VII strains can be adapted to grow in eggs.	63
4. The WW strain does not cause disease in Lewis Mai/f rats (Stroop, personal observation)	
5. No strain has been demonstrated to grow in rabbits, monkeys, or Guinea pigs.	

[a]The TO and GD VII strains were originally thought to have a biphasic pH stability curve (stable at pH 3.3 and 8.0), when incubated at 37°C in the phosphate-glycine-acetate buffer of Northrup and DeKruif (326). It was later shown that the pH stability curves for picornaviruses were subject to great variation due to the presence or absence of 0.1 M cloride or bromide ion and other variables of buffer composition (cf. Rueckert, ref. 279).

adults in the population will have developed an immunity to the type of poliovirus endemic within that population. The introduction of modern standards of community health during the late 1800s lead to a change in the age incidence of primary infections by limiting the spread of poliovirus throughout the community. Thus, many children did not acquire immunity by the time they reached adolescence or adulthood. Primary infection of adolescents is very much more likely to result in severe paralytic disease than primary infection of very young children. The epidemics observed at the turn of the century are attributed to the decreased number of immune individuals in developed communities (83).

Theiler's virus infection, whether experimental or spontaneous, follows a very similar pattern of age susceptibility (63,177,231,324,325). Suckling mice are exquisitely susceptible to acute encephalomyelitis when infected with Theiler's virus by the intracerebral route. Increasing resistance to infection develops rapidly from birth up to about six or seven weeks of age, then develops more slowly thereafter (325).

Several investigators have researched the apparent age dependent susceptibility of mice to experimental

Theiler's virus infection. Olitsky was able to show that Theiler's viruses could be isolated from the feces of normal and experimentally inoculated mice (231). When uninoculated pregnant mice were housed with infected mice, Olitsky demonstrated that virus could not be isolated from fetuses or from suckling mice up to 12 days post partum, but after this period, virus could be routinely isolated from the intestinal contents, the intestinal mucosa or the washed intestinal walls of normal mice. He further demonstrated that virus could not be found in the fetus even if the mother bearing the litter was experimentally infected with Theiler's virus. Presumably the absence of virus in neonatal mice is due to maternal antibody. Liu, et. al. (177) and Parker, et. al. (239) were able to demonstrate that between 82 to 100% of all mice tested from mouse colonies in the United States had developed antibodies in their serum by about 11 to 22 weeks of age, yet showed no sign of spontaneous disease. These studies showed that Theiler's viruses are endemic to mouse colonies and that mice apparently develop antibodies due to a subclinical epizootic infection. Antibody protection apparently accounts for the very low (1/1000-1/5000) incidence of spontaneous paralysis due to Theiler's virus (325). Only the occasional adult mouse is

susceptible to paralytic disease.

These experiments taken together illustrate several salient features of Theiler's virus infection. First, like poliovirus infection of humans, there appears to be an age dependent susceptibility to infection with young individuals being more susceptible to infection than old individuals. Second, the relative resistance of older individuals within a population to poliovirus infection is dependent on immunity acquired earlier in life which also appears to be the case with Theiler's virus. Thirdly, it demonstrates that these viruses are normal enteric viruses of mice as poliovirus is a normal enteric virus of humans (52). Furthermore, it was demonstrated by Olitsky that Theiler's virus is able to persist in the gut of an animal after acquiring the infection at about 12 days post partum. The virus persists until about 6 months of age when viral titers in feces, intestinal content, mucosa and washed intestinal walls begin to decline. This is similar to the "transient persistence" following oral administration of poliovirus to humans (23,285). These experiments established many similarities between Theiler's virus infection of mice and poliovirus infection of humans with regard to the infectious agents and the pathogenesis of the

agents in their respective hosts.

One of the issues of the day in the late 1940s and 1950s was the question of how poliovirus enters the central nervous system following peripheral inoculation through inhalation or injestion of the virus. Virus was believed to replicate in extraneural tissues at or adjacent to the site of inoculation. Once sufficient viral titers had been achieved in extraneural sites, one point of view held that the virus infected nerve endings and gained access to the central nervous system by retrograde ascent along nerve fibers (285). Within the central nervous system the virus was thought to home to, and replicate in, the anterior horn cells of the spinal cord. The other view held that once virus had replicated in extraneural tissues to a sufficiently high titer it spread to the draining lymph nodes, then to the blood, and through the resulting viremia gained access to the central nervous system via certain limited, presumably modified, vascular zones (23). From its point of entry into the central nervous system, the virus was envisioned to spread along specific neural pathways and home to the anterior horn cells.

It was clear to all of the investigators that poliovirus was able to replicate in extraneural tissues in the human and the experimentally infected chimpanzee.

The sites of extraneural replication were primarily the tonsils and the Peyers patches, which are nonencapsulated lymphoid tissues present in the wall of the small intestine, and to a lesser extent in the lymph nodes and brown fat (23,293). The central question that needed to be answered to resolve these two points of view was whether or not a viremia had to precede the onset of paralysis. Experimental evidence was sought with Theiler's virus infection of mice to determine the route of entry of virus into the central nervous system. These studies were hampered by the relatively poor neuroinvasiveness of most of the strains of Theiler's virus used for these studies. Only rarely was virus demonstrated to invade the central nervous system following peripheral inoculation of weanling mice with a Theiler's original isolate. The early studies by Theiler and Gard (326,327) failed to demonstrate a viremia following peripheral inoculation of the original strains into weanling mice. Only the very neurotropic GD VII and FA strains of Theiler's virus had been demonstrated to invade the central nervous system following peripheral inoculation (Table 2). More recent studies with some of Theiler's original isolates and the DA strain (see below) by Lipton (167) have also

failed to demonstrate the presence of virus in blood during the course of infection. Therefore, these viruses were not demonstrated to cause a viremia prior to the onset of paralysis.

The question of spread of poliovirus through the central nervous system by direct neural ascent or following a viremia was ultimately solved using fluorescent antibody staining methods by following the course of infection of cynomolgus monkeys infected orally with poliovirus type 1 (140). Briefly, specific fluorescence was seen first in pharyngeal epithelial cells, later in submucosal cells of the oropharynx, followed by cells of the lower alimentary tract. Foci of fluorescence were seen next in local lymph nodes, and after viremia was established, fluorescence was detected in the macrophages of spleen and liver. Fluorescent cells were only found in the central nervous system of animals suffering paralysis following the viremia. Virus is therefore thought to gain entry into the central nervous system following viremia (219) although some laboratory strains of poliovirus have been demonstrated to invade the brain directly by the neural route (218,285).

Using the immunofluorescence technique to follow the pathogenesis of Theiler's encephalomyelitis infection in mice, Liu, et. al. (177) were able to demonstrate

the distribution of virus specific antigen in tissues throughout the course of infection. These investigators found that specific fluorescence correlated well with infectious virus titers and progress of the infection following intracerebral inoculation. When the same infectivity studies were repeated by inoculating the virus peripherally or feeding the virus to mice, low titers of virus were found in visceral organs including heart, lung, liver, spleen, pancreas, stomach, ileum and colon as well as brain and spinal cord. However, when immunofluorescent studies were performed on tissues taken from mice inoculated peripherally, specific fluorescence was not detectable in any organ except brain. These authors could not account for the apparent discrepancy between the absence of specific fluorescence in visceral organs and the presence of titratable virus in the same organs except to suggest that the low levels of titratable virus found may have reflected low levels of replication resulting in little expression of viral antigens. Therefore, the site of extraneural replication of Theiler's viruses and the pathogenesis of invasion of the central nervous system of mice following peripheral inoculation of these agents, remains unresolved.

In 1949 Enders, Weller and Robbins demonstrated the growth of poliovirus in cultures of non-neural tissues (83). This fundamental discovery allowed investigators to work on polioviral biology in a tissue culture system without having to use monkeys. Also, in the late 1940s, the United States National Foundation for Infantile Paralysis organized a nationwide door knock campaign imaginatively called the "March of Dimes." The purpose of the March of Dimes was to raise funds, allowing the foundation to sponsor a massive research drive to eradicate poliomyelitis by development of a poliovirus vaccine. With funding so available and Enders' recent discovery, it was a short time before Jonas Salk developed the formalin inactivated, and Albert Sabin, Hilary Koprowski and other developed the live attenuated oral viral vaccines. With vaccines available the incidence of poliomyelitis declined rapidly; even in Sweden, where paralytic poliomyelitis first became apparent in epidemic form, the Salk vaccine erradicated the disease. Subsequent to the introduction and widespread use of poliovirus vaccines, interest in studying Theiler's virus infection in mice declined.

Just prior to the introduction and licensing of the Salk vaccine in 1954, Daniels, et. al. (63) published a report of a newly isolated strain of Theiler's virus, the DA strain, which exhibited a broader spectrum of biologic activities in vivo than the GDVII, FA and TO strains of Theiler's virus (Table 1, Table 2, Table 3). Following intracranial inoculation of the DA strain of Theiler's virus, mice developed flaccid hind limb paralysis characteristic of the TO strains of Theiler's virus (Table 2). Some of the mice died during this acute disease phase of encephalitis. Those mice which survived the acute phase of disease and regained some function of their paralyzed hind limbs developed, several weeks after inoculation, a peculiar spastic hind limb paresis which imparted to animals so affected, a characteristic waddling gait (63). Histologically, lesions occurred during the acute phase, in spinal cord and brain and looked identical to lesions observed in earlier studies following intracranial inoculation of the TO strains of Theiler's virus. However, what made the DA strain interesting is that it had the capability of inducing a chronic form of the disease attended by marked myelin destruction of the spinal cord (Table 3). The chronic phase followed the acute phase. The demyelinating lesions occurred in the lateral,

ventral and occasionally posterior columns, however, the dorsal root ganglia were never affected. A viremia was detected prior to the onset of the neurologic signs in suckling but not weanling mice inoculated intracranially with the virus (Table 2, Table 3). Although Daniels, et. al. reported their findings concerning this new isolate of Theiler's virus in 1952 and described a new type of disease which could be induced by a strain of Theiler's virus, little attention was given to these studies by other investigators in the field. This lack of interest can only be explained in view of the push by the scientific community to eradicate poliovirus infection worldwide.

Most investigations of Theiler's virus pathogenesis have resulted in elucidation of its behavior and pathologic effects in the central nervous system. Depending on the strain of virus and route of inoculation, however, Theiler's virus may also infect and cause disease in other tissues, most notably muscle. Rustigian and Pappenheimer induced myositis in young mice inoculated intramuscularly with the GDVII and FA strains of Theiler's virus (284) (Table 2). Daniels confirmed these observations in 1952 (63) and extended them to include the TO and DA strains (Table 2). Following intracranial inoculation,

however, myositis was produced in weanling mice with only the TO strains (63) (Table 3).

Many other viruses of several genera had also been shown to induce myositis. The Coxsackie viruses, Types A and B, (82,83) SK and Jungeblut, lymphocytic choriomeningitis, herpes simplex, HF strain, Eastern Equine Encephalitis and JHM viruses had been shown to cause myositis in neonatal mice following intramuscular injection in young mice (284). Incidentally, the Coxsackie viruses have been linked to human disease such as meningitis, macular and vesicular rashes associated with "summer minor illnesses," vesicular pharyngitis, epidemic myalgia, carditis, hemolytic uremia and pancreatitis (82,83).

A unique feature of Theiler's virus infection of mice which gained little attention at the time was that these viruses have an ability to persist in central nervous system tissues. Theiler demonstrated viral persistence following intracranial inoculation as early as 1937 (324), although he did not report whether persistently infected animals also exhibited the chronic demyelination observed by Daniels, et. al. (Table 2, Table 3). Daniels, et. al. reported the persistence of virus through the chronic phase of the disease following

intracranial inoculation of the DA strain of Theiler's virus and found that the virus could be isolated directly from central nervous system tissue up to 90 days post-infection (63). Persistence has also been demonstrated recently following IC inoculation of the DA strain of Theiler's virus by Dal Canto and Lipton (60). Persistence of virus occurs in spite of a high humoral immune response (60,67) throughout the animal's life. This feature of Theiler's virus infection is in contrast to poliovirus infection of humans; indeed it is unlike all other picornaviral infections of animals studied to date. The mechanisms of persistence and the relationship of persistence to chronic disease are yet to be determined.

There were relatively few reports in the literature about Theiler's virus infection of mice from the mid 1950s to 1975 when Howard Lipton examined DA virus infection of weanling mice and described the pathogenesis and histopathology of the disease (167). In these studies Lipton was able to demonstrate a clinical course of disease following intracranial inoculation of DA virus into weanling mice which was identical to what had been shown by Daniels, et. al. 27 years earlier. Briefly, experimental animals developed paralysis nine

to eighteen days after inoculation and approximately fifty percent died during the acute illness. Those survivors with minimal paralysis recovered within one to two weeks, although mice severely paralyzed did not appreciably improve. Approximately 45-60 days after infection, survivors began to develop the spastic waddling gait (167,172) characteristic of chronic disease (63). The virus replicated in the central nervous system of infected animals reaching maximal titers approximately coincident with the development of paralysis and then declined rapidly over the next week. Antibody titers in serum began to rise two weeks after infection and steadily increased thereafter. At no time was virus detectable in blood. By immunofluorescence virus antigen could be demonstrated for two weeks after infection in scattered cells in the hypothalamus, thalamus, brain stem and spinal cord. During the chronic phase of the disease, viral antigens, if detected at all, were found only in the spinal cord. Histologically, the acute phase was identical to poliomyelitis in humans and monkeys, and was characterized as an acute polioencephalitis with neuronal necrosis and neuronophagia, the most common observations. Indeed, acute paralytic phase of the disease has been

appropriately termed a "gray matter disease" in that few histopathologic changes can be observed in white matter during this period.

During the chronic phase, nearly an opposite histopathologic inflammatory change was noted. Inflammatory changes were confined predominantly to spinal cord; only rarely were gray matter lesions observed. The inflammatory response was confined primarily to the white matter and consisted of pronounced parenchymal infiltrates and perivascular cuffs of mononuclear cells, some macrophages and what appeared to be reactive astrocytes. Intense leptomeningeal infiltrates were also observed. The surrounding neuropil appeared microcystic, however, blood vessels remained intact. Silver stains of longitudinal sections revealed demyelinated lesions with relative preservation of axis cylinders (167). In large and apparently older lesions, conspicuous gliosis was also present (167).

Dal Canto and Lipton examined the ultrastructure of Theiler's virus infection and concluded that myelin breakdown during the chronic phase of disease was due to mononuclear cells stripping myelin lamellae from axons (60,61,62). Low levels of persistent virus could be recovered from central nervous system tissue homogenates from the beginning of the chronic phase, approximately

22 days post infection, until as late as 210 days post-infection. Remarkably, these investigators were not able to demonstrate viral inclusions during the chronic phase of disease by electron microscopy. It seems likely that the failure to observe paracrystalline arrays in central nervous system tissues during the chronic infection was a problem of examining enough tissues from the appropriate phase of cytolytic infection. Oligodendrocytes in the vicinity of demyelinating lesions showed no degenerative changes. The demyelination occurred first at 15 days post-infection and lasted throughout the chronic stage of disease. Very late after infection numerous plasma cells could also be observed. Because these investigators were not able to demonstrate viral antigens during the chronic demyelinating phase of disease, did not observe any degenerative changes in oligodendroglial cells, and were unable to demonstrate viral inclusions in any brain cells, the possibility that the demyelination was immune-mediated became apparent.

Lipton and Dal Canto investigated the effect of immunosuppression on demyelination by treatment of infected mice with cyclophosphamide and rabbit anti-thymocyte serum (173). In these experiments, weanling

mice were inoculated intracerebrally with the DA strain of Theiler's virus followed by intraperitoneal inoculations of the immunosuppressive agents on days 3, 8, 12 and 17 post-inoculation. At 17 to 22 days post-inoculation, mice were sacrificed and central nervous system tissues were examined. In control mice, which had received no immunosuppressive treatment, 100 percent of the animals had parenchymal inflammatory responses in their spinal cords. The majority of the inflammatory cells were lymphocytes or monocytes, although plasma cells could also be identified. In those animals which received the cyclophosphamide treatment, only 7 percent exhibited a cellular inflammatory response and none of the animals which had received antiserum to mouse thymocytes had cellular inflammatory response. The reduction in the cellular inflammatory response observed in mice which had received the immunosuppressive treatment correlated with an absence of demyelination (173). Interestingly, the microglial response in brain was enhanced by both immunosuppressive treatments (174). These data strongly suggested that the demyelination observed during the chronic Theiler's virus infection in mice was immune-mediated (173). Lipton and Dal Canto (174) further demonstrated that the immunosuppressive treatment of

Theiler's virus infected mice resulted in an increase in mortality which correlated with an increase in gray matter disease during the chronic phase. They concluded that immunosuppression potentiated cytolytic infection in neurons by abrogating humoral immunity, resulting in an increase in mortality (174).

The studies of Lipton and Dal Canto (167,173,174) suggested that chronic demyelination was immune mediated. An alternative explanation was that the virus was capable of infecting and replicating in oligodendrocytes and upon immunosuppressive therapy, the progeny virus from these cells was capable of dissemination to neurons adjacent to the infected oligodendrocyte and initiating a new round of neuronolytic infection (ie, potentiating polioencephalitis). Indirect evidence to support this postulate came from two observations. Penney, et. al. (245) were able to demonstrate paracrystalline arrays in both neurons and oligodendroglia of suckling mice infected with the recently isolated WW strain of Theiler's virus (348) (Table 1). This particular strain of Theiler's virus is much like Theiler's original strains of virus (169) (Table 2 and 3). Furthermore, Wroblewska, et. al. were able to demonstrate paracrystalline arrays in neurons, oligodendroglial cells and astrocytes in organotypic cultures of mouse cerebellum

(349). These investigators concluded that demyelination was secondary to the lysis of the oligodendrocytes by the virus. Collectively, these studies indicated that the possibility existed for viral growth in oligodendrocytes, even though Lipton, et. al. had been unable to observe viral crystalline arrays during chronic disease. Thus it was at least possible that the demyelination observed during the chronic phase of disease was due to a direct lysis of infected oligodendrocytes followed secondarily by a loss of myelin.

Until 1975 experiments with Theiler's virus infection in mice were hampered by the fact that Theiler's virus had not been adapted to tissue culture. Without tissue culture adaptation of the virus, viral titrations of various organs had to be carried out by subinoculation of organ homogenates into other mice. Furthermore, experiments designed to elucidate the serologic relationships of the various Theiler's virus strains which had been identified also had to be carried out in animals. These experiments involved either inoculation of mice with sublethal doses of one strain of Theiler's virus followed by inoculation of a different strain of virus and determination of cross-neutralization between the two strains or incubation of one strain of virus with antisera raised in mice directed against another strain

of virus and inoculation of the immune complex into mice. Such experiments were time consuming, laborious and expensive. In 1975 Lipton described the successful adaptation of the DA strain (167) and in 1978, the GDVII, TO4 and WW strains (169) to tissue culture. The adaptation process was defined as complete when cytopathic effect (CPE) was produced. It should be pointed out that the brain-derived Theiler's viruses, with the exception of GDVII, do not produce CPE when grown directly on tissue culture cells, and require "blind-subpassage". By preparing monospecific antisera in guinea pigs to each of the tissue culture adapted strains, Lipton was able to demonstrate that all of the viruses were related to each other (169) and that no serological subgroups exist as is the case for human polioviruses (158,162). Interestingly, Lipton was also able to demonstrate that bovine serum, a normal constituent of tissue culture media, contained IgG which could neutralize Theiler's viruses but not the unrelated picornavirus, encephalomyocarditis virus (169).

Lipton also demonstrated a difference in plaque size between the TO strains (DA, TO4, WW) and the GDVII strain of Theiler's virus. He found that the TO strains

produced small plaques 1 mm or less in diameter and that the GDVII strain produced both small and large plaques with the large plaques being approximately 3-5 mm in diameter (169). When the DA, WW and TO4 tissue culture adapted strains of Theiler's virus were inoculated intracranially into suckling mice, Lipton and Dal Canto found that virus reisolated from CNS tissue was capable of producing direct cytopathic effect in tissue culture (175). However, it is important to note that following tissue culture adaptation of the WW, DA and TO4 strains, the ability to produce early acute flaccid hind limb paralysis in suckling or weanling mice following intracranial inoculation was lost (175) (Table 5).

Intracerebral inoculation of mice with the tissue culture adapted TO strains of Theiler's virus resulted in the production of the chronic disease without the antecedent early disease (168,170,175). The period from incubation until first onset of neurologic disease varied from 11 to 22 weaks following intracranial inoculation of the DA, WW or TO4 strains of Theiler's virus in Swiss mice (Table 5). The clinical signs of mice becoming ill after this prolonged incubation period was the spastic waddling gait characteristic of chronic disease. The clinical appearance correlated

histologically with demyelinated lesions in the spinal cord; inflammatory cells present in these lesions were mainly lymphocytes and macrophages. The disease, when viewed from clinical and histopathologic standpoints, was identical to chronic disease seen after intracranial inoculation of brain-derived stocks of DA virus (175).

Interestingly, although animals did not have the clinical manifestations of the acute phase of disease, i.e., flaccid hind limb paralysis, virus was replicating in the central nervous system during the period when flaccid hind limb paralysis would have been observed if brain-derived stocks of virus were used (168,170,175). Following acute replication in CNS tissues without production of disease, virus titers begin to decline in both brain and spinal cord, becoming barely detectable in brain, yet remaining at low but significant levels in spinal cord throughout the remainder of the animal's life. In contrast, when tissue culture stocks of GDVII or FA virus were inoculated intracerebrally into weanling mice, they died of acute encephalitis 5 to 7 days after infection (168,170) (Table 5). When progeny virus was isolated from mice inoculated with tissue culture adapted GDVII or DA viruses, and recultured in tissue culture, the viruses maintained their plaque size and morphology.

TABLE 5

IN VIVO AND IN VITRO BIOLOGICAL CHARACTERISTICS OF TISSUE CULTURE ADAPTED STRAINS OF THEILER'S MURINE ENCEPHALOMYELITIS VIRUSES

STRAIN	PLAQUE SIZE[a]	ABILITY TO ESTABLISH PERSISTENCE	NEUROVIRULENCE[e] (pfu/LD_{50})
DA	small[b,c,d]	+[b]	low[b,d]
TO- IV	small[b,c,d]	+[b]	low[b,d]
WW	small[b,c,d]	+[b]	low[b,d]
Yale	small[b,c,d]	+[b]	low[b,d]
BeAn8386	small[b]	+[b]	low[b]
GD VII	large[b,c,d,g]	-[b]	high[b,d]
FA	large[b,c,d,g]	-[b]	high[b,d]

(CONTINUED)

TABLE 5 (CONT'D)

IN VIVO AND IN VITRO BIOLOGICAL CHARACTERISTICS OF TISSUE CULTURE ADAPTED STRAINS OF THEILER'S MURINE ENCEPHALOMYELITIS VIRUSES

STRAIN	TYPE OF DISEASE PRODUCED	FIRST ONSET OF SIGNS	DAY OF MAXIMUM RECOVERABLE VIRAL TITERS IN CNS
DA	chronic demyelination[b,d]	10-14 weeks[f,d] pi[i]	5 days[b] pi
TO -IV	chronic demyelination[b,d]	10-15 weeks[f,d] pi	NR[h]
WW	chronic demyelination[b,d,j]	10-22 weeks[f,d] pi	NR
Yale	chronic demyelination[b,d]	NR	NR
BeAn8386	chronic demyelination[b,d]	NR	NR
GD VII	acute encephalitis[b,d]	5 - 9 days[f] pi	NR
FA	acute encephalitis[b,d]	NR	NR

[a]Large plaques were 2-5 mm in diameter; small plaques were < 0.5 mm (169).

[b]Ref. 170

[c]Ref. 169

[d]Ref. 168

[e]Relative virulence determined by the number of pfu required to produce death (pfu/LD_{50}). For DA pfu/LD_{50} = 100; for GD VII, pfu/LD_{50} = 1.0 (170).

[f]Ref. 175

[g]Lipton first reported the presence of both large and small plaques in L cells infected with GD VII (169), but later stated that only large plaques were produced by plaque purified GD VII grown in BHK-21 cells (168, 170); however, some "intermediate" sized plaques were seen even in such purified stocks of GDVII (vide., Fig. 2, ref. 170).

[h]not reported

[i]Post-Inoculation

Further studies by Lipton demonstrated that persistence in CNS tissue could only be established using the relatively avirulent, small plaque strains of Theiler's virus (tissue culture TO strains) which produced no illness during the first month after inoculation, but not with the highly virulent large plaque strains, GDVII and FA viruses. Lipton concluded from these observations that the ability to establish persistent infections in mouse CNS tissues with the viruses which had been adapted to tissue culture depended on the plaque size of the virus in question (170). The available data on the in vivo and in vitro properties of the tissue cultured strains of Theiler's viruses are summarized in Table 5.

Having identified strains of tissue culture adapted Theiler's viruses that differed regarding their ability to establish persistence, Lipton and his colleagues examined the ultrastructural replication of these viruses in tissue culture cells. Friedmann and Lipton compared the replication of tissue culture DA and GDVII viruses and found that GDVII virus formed large paracrystalline arrays in infected cells (91). In contrast, DA virus did not form crystalline arrays at the same stage of infection. Instead, viral particles were found in non-crystalline, membrane-associated forms in the

cytoplasm of infected cells. The contrast between the morphogenesis of DA and GDVII viruses appeared to correlate with the rate of replication in and release of progeny virus from tissue culture cells. GDVII virus produced a large amount of extracellular virus whereas the DA virus was essentially not released from infected cells into the medium (91). Although the differences between these two viruses during the later stages of infection were striking, it was unclear what the differences in morphogenesis correlated with in terms of their pathogenetic behavior in vivo. Attempting to gain further insight into the differences between the tissue culture adapted, highly virulent GDVII and relatively avirulent DA viruses, Lipton and Friedmann examined some of the physical properties as well as the structural polypeptide composition of seven strains of tissue culture adapted Theiler's virus which were similar in their biologic behavior in vivo to GDVII or DA viruses (172).

Purified preparations were made of the highly virulent GDVII and FA strains. These strains produce a rapidly fatal encephalitis in mice, form large plaques in cell culture and had been shown to be unable to establish persistent infections in mice. Purified

virus preparations were also made of the DA, WW, TO4, Yale and BeAn 8386 strains. These strains had been shown to be approximately 1000 fold less virulent than the FA and GDVII strains, form small plaques in tissue culture and were capable of producing a persistent infection in vivo resulting in the chronic inflammatory demyelinating disease. All virus preparations were characterized biochemically to elucidate the correlates between their molecular structure and their pathologic potential and morphology in tissue culture (176).

All viruses were found in isopycnic cesium sulfate gradients to band at a buoyant density of 1.34 g/cm^3. Electron microscopy revealed that all viruses had an average diameter of 28 nm, thus by size and isopycnic density, all viruses were morphologically identical. Viruses which had been radiolabelled in tissue culture prior to purification were subjected to discontinuous sodium dodecyl sulfate-polyacrylamide gel electrophoresis to determine the molecular weights of the structural polypeptides. As a control, purified poliovirus type 1 was co-electrophoresed with the Theiler's viruses. They found that the Theiler's viruses contained four structural polypeptides which is characteristic of all

picornaviruses (see below). It was found, however, that the VP1 polypeptide was slightly larger and the VP2 polypeptide was slightly smaller for the highly virulent GDVII and FA strains compared to the same polypeptides in the less virulent strains (Table 4). Moreover, Lipton and Friedmann demonstrated that the less virulent viruses, when incubated with trypsin at a concentration of 0.5 mg/ml for 10 minutes at 37^{o}C, preferentially lost an approximate 2000 molecular weight fragment of the VP1 polypeptide resulting in co-migration of the VP1 and VP2 polypeptides of the viruses in polyacrylamide gels (176). The same trypsin sensitivity of the VP1 polypeptide was not observed with the highly virulent FA and GDVII viruses. These studies suggested that certain "biochemical markers" existed to distinguish the highly virulent from the relatively avirulent strains of tissue culture adapted Theiler's viruses (176).

At the same time Lipton and Friedmann were investigating the tissue culture adapted strains of Theiler's viruses, work was undertaken to describe the properties of Theiler's virus isolated directly from acutely infected central nervous system tissues of mice. A portion of the results presented in this study will

describe the biophyscial and biochemical properties of this virus.

Ignoring the differences between tissue culture adapted viruses and brain-derived viruses for the moment, the studies reported here and the studies by Lipton and Friedmann offer the first polypeptide characterizations of Theiler's viruses and firmly establish that the Theiler's viruses fulfill the necessary criteria for their inclusion in the genus Enterovirus and family Picornaviridae (52).

Theiler's Viruses Defined as Picornaviruses: Up until the report by Lipton and Friedmann (176), no complete proof that the Theiler's viruses belonged in the Picornaviridae had been put forth in the literature. Several suggestive lines of evidence, however, clearly indicated that these viruses were picornaviruses. First, it was clear from the early work by Theiler and Theiler and Gard (323,324,326,327) that these viruses had several of the physical and chemical properties in common with poliovirus, the most widely studied picornavirus. The pH stability of the virus was characteristic of poliovirus and the other enteroviruses (326) not the cardioviruses, a related genus (305) (Table 4). Theiler's viruses were shown to be non-enveloped by their stability in ether

(326) and fluorocarbon (89), and like poliovirus were destroyed by treatment with high concentrations of hydrogen peroxide (326). These viruses were shown to be serologically related to each other as has been mentioned above (169) but were serologically distinct from poliovirus (324,325,327), Yellow Fever virus (326) and the cardioviruses (42,146). The Theiler's viruses were shown, however, to share complement fixing antigens with the cardioviruses and another serotype, Vilyuisk virus (42,146). This is not unlike the situation with the three serologic types of poliovirus, Type 1, 2, and 3. Furthermore, the size of the Theiler's viruses was determined to be approximately the same size as poliovirus (324, 326). Although these initial sizing experiments were in error yielding a size between 9 and 13 nm, they nonetheless showed that the Theiler's viruses and poliovirus were similar. These observations tentatively suggested that Theiler's viruses were structurally probably very similar to poliovirus. Moreover the fact that the Theiler's viruses and poliovirus were predominately neurotropic and produced clinically and histologically identical diseases in their natural hosts gave further credibility to the assignment of Theiler's viruses to the Picornaviridae. It was not

until 1959 that the nucleic acid of Theiler's viruses was documented to be RNA by its sensitivity to RNAse, its resistance to DNAse and by the fact it could be precipitated in solutions of sodium chloride under conditions in which only RNA precipitates (89). Thus, it was clear that Theiler's viruses were a group of small (-pico), RNA containing viruses.

With the report that poliovirus could be adapted to non-neural tissue culture by Enders, et. al., it became possible to examine large quantities of poliovirus (29,165) and elucidate many more characteristics of the virus; indeed the picornaviruses are among the best studied RNA animal virus to date and it is therefore germane to review the structure, antigenic characteristics and morphogenesis of picornaviral molecular biology.

Structure, Antigenicity and Morphogenesis of Picornaviruses:

The vertebrate picornaviruses consist of a large number of agents which have been classified according to their physical and chemical properties, host range, and serologic relatedness (279,295). Table 6 illustrates the classification of the four genera of the family Picornaviridae. The chief distinction between the enteroviruses and the cardioviruses lies in their stability at different pHs. The enteroviruses are

TABLE 6

SOME PHYSICAL AND BIOLOGICAL PROPERTIES OF PICORNAVIRUSES[a]

I. PHYSICAL PROPERTIES

A. pH Stability

1. Genus Enterovirus: stable at pH 3-10
2. Genus Cardiovirus: stable at pH < 5 and > 7
3. Genus Rhinovirus: stable at pH 6.8-7.3
4. Genus Aphtovirus: stable at pH > 6.5

B. Effect of Solvents

1. Stable in ether and fluorocarbon
2. Stable in deoxycholate
3. Destroyed by H_2O_2

C. Effect of Dessication:

1. Destroyed by drying

D. Effect of Temperature:

1. Rapidly destroyed by heating at 50^{o} C.

E. Size:

1. 26 - 30 nm

F. Nucleic Acid:

1. 35 S single stranded
2. RNA
3. 2.6 X 10^6 dalton mol wt

G. Density in Cesium Soultions

1. Genus Enterovirus: ~ 1.34 gm/cm^3
2. Genus Cardiovirus: ~ 1.34 gm/cm^3
3. Genus Rhinovirus: ~ 1.40 gm/cm^3
4. Genus Aphtovirus: ~ 1.43 gm/cm

H. Sedimentation Velocity:

1. 151 - 162 S ($S_{20^{\omega}}$)

I. Polypeptide Composition:

Polypeptide Mol Wt x 10^3 daltons				
VPO (ε)	VP1 (α)	VP2 (β)	VP3 (γ)	VP4 (δ)
~40	28-36	26-32	21-30	~6-14

(CONTINUED)

TABLE 6 (CONT'D)

SOME PHYSICAL AND BIOLOGICAL PROPERTIES OF PICORNAVIRUSES

II. BIOLOGICAL PROPERTIES

A. Serology

1. Genus Enterovirus: Poliovirus (serotypes I, II, III)
Coxsackie A (23 serotypes)
Coxsackie B (6 serotypes)
Echoviruses (31 serotypes)

There are numerous sub-types of the Coxsackie and Enteroviruses; the original distiction between these subgroups was the ability of the former to grow in baby mice. No such distinctions are now being made. No cross neutralization exists between sub-types.

2. Genus Cardiovirus: Encephalomyocarditis virus (EMC)
Maus-Elberfeld (ME)
Columbia-SK
MM

These strains are probably all members of a single serotype, and will evoke cross neutralizing antibodies *in vivo*.

3. Genus Rhinovirus: Hundreds of serotypes

4. Genus Aphtovirus: Foot-and-Mouth Disease Virus (FMDV) (6 serotypes)

B. Host Range:

Picornaviruses have been isolated from animals as diverse as caterpillars (Gonometa virus), fish, pigs, sheep, cattle, horses and man. Poliovirus infects man as its natural host, but can be adapted to grow in monkeys (see text), and one strain (Lansing) is pathogenic for mice. Equine rhinoviruses have a broad host range including rabbits, man, monkeys and Guinea pigs. Coxsackie viruses can grow in man and animals.

[a]Compiled from References 162, 279 and 295.

stable at pH 3-10 whereas the cardioviruses have a biphasic pH stability curve. Viruses belonging to both of these genuses sediment at a buoyant density of 1.34 g/cm^3 in cesium chloride solutions. This feature distinguishes these two genuses from the rhinoviruses which sediment at a buoyant density of 1.40 g/cm^3 and the aphtoviruses which sediment at a buoyant density of 1.43 g/cm^3. The difference in buoyant density between the entero- and cardioviruses (1.34 g/cm^3) and the rhino- and aphtoviruses (1.40 - 1.43 g/cm^3) has been postulated to be due to the difference in penetration of cesium ions and subsequent reaction with the RNA (28,194,195). This postulate is supported by the fact that the density of empty capsids (the RNA-free protein shells of these viruses) of the rhinoviruses and enteroviruses have similar buoyant densities in cesium chloride solution (278). Accepting values for the density of empty capsids as 1.30 g/cm^3 and of RNA as 1.91 g/cm^3, the theoretical density of a particle containing approximately 30% RNA and 70% should be 1.48 g/cm^3. Thus, if RNA and protein in an intact virion react completely with cesium ions, the density of a picornavirus should be 1.48 g/cm^3. Since in fact, the density of the entero- and cardioviruses is 0.14 g/cm^3 lighter than the theoretical maximum, it is clear that the RNA

in the entero- and cardioviruses is protected from reaction with the cesium ions by the relative impermeability of the protein capsids.

All of the picornaviruses studied thus far are composed of a molecule of single stranded (+) RNA which comprises 30% by weight of the total molecular weight of the virus ("+" denotes the same polarity as cellular mRNA) (6,8,30,80,164,202,208,270,283,288,292) with the remaining 70% by weight of the virus being comprised of protein. There is no evidence for the presence of glycoprotein or lipid in the virion (31). The molecular weight of the RNA ranges between 2.4 to 2.8 million daltons with a mean of 2.6 million daltons by sedimentation analysis, electron microscopy and by migration in polyacrylamide or methyl-mercury agarose gels (94,183,223, 224,317,258).

Initial studies on the structure of viral RNA revealed one striking difference between viral RNA and eukaryotic mRNA. The 5' termini of nearly all eukaryotic mRNA are capped (297). The cap structure consists of two nucleotides linked by 5' - 3' orthophosphate linkages to the terminal ribose of the message. The distal nucleotide always contains an 0-methyl group in the 2' position of the ribose and the proximal nucleotide may have the 2' 0-methyl group in certain cases. A guanylate

residue methylated at the seventh position (m^7G) of the purine is attached by a 5'-5' triphosphate linkage to the distal 2' 0-methylated nucleotide. This 5'-m^7G cap is absent from picornavirus RNAs; instead the 5' terminus appears to end in pUp (118,221).

Covalently linked to the pUp is a 4000 dalton molecular weight protein termed VPg (161, 287). The VPg protein has been detected on the 5' termini of nascent (+) and complementary (-) RNA isolated from infected cells (81) (Fig. 1). The function of VPg has not been completely determined. However, it is possible that it may be necessary to prime initiation of transcription of the nascent (+) strands of RNA by the viral replicase (222). The VPg protein is apparently enzymatically cleaved from poliovirion RNA destined to serve as viral mRNA (4) (Fig. 1).

The 3'-end of picornaviral RNA and most eukaryotic cellular mRNAs consists of a long homopolymeric sequence of polyadenylic acid (351). The poly(A) sequence is required for the infectivity of polioviral RNA (300).

The protein component of a picornavirion is composed of four non-identical polypeptide chains (32,185,186,188, 189,277,280). The polypeptide chains in order of decreasing molecular weight have molecular

weights on the order of 28 to 36 thousand daltons (VP 1, α), 26 to 32 thousand daltons (VP 2, β), 21 to 30 thousand daltons (VP 3, γ) and 5.5 to 14 thousand daltons (VP 4, δ). Occasionally traces of a fifth polypeptide with a molecular weight of about 40 thousand daltons (VP 0, ε) are observed. The VP 1, VP 2, VP 3, VP 4, VP 0 nomenclature has been adopted in accordance with the designation employed for poliovirus (164) and the α, β, γ, δ, ε nomenclature is in accordance with the chain designation adopted for certain cardio- and rhinoviruses (279).

The RNA and its protein capsid together form the virion, which has been shown to have a sedimentation coefficient ($S_{20}\omega$) of 150 to 160 S (279,295). When poliovirus was crystallized by Schaeffer and Schwerdt (288), Finch and Klug (85), subjected the crystals to x-ray diffraction analysis and concluded that the structure of the picornavirus was a regular solid with five fold symmetry around a point, three fold symmetry around a face and two fold symmetry along an edge; this symmetrical pattern could be best visualized as a regular icosahderon. Crick and Watson (58) proposed that the picornavirion was made up of sixty identical asymmetric structural units. However, analysis of turnip yellow mosaic virus (TYMV), effected by high resolution electron microscopy, revealed that TYMV was organized into thirty-two "structural units" (capsomeres), not sixty. These thirty-two capsomeres were found by Finch and Klug

(86) to be composed of 12 clusters of five chains and 20 clusters of six chains. This evidence, plus evidence obtained with other picornaviruses, indicated that the thirty-two capsomere model possessing 5:3:2 symmetry was indeed correct. Therefore picornaviruses are generally regular icosahedrons possessing 5:3:2 symmetry with a 60 n subunit model (12 pentamers plus 20 hexamers equals 180; n = 3). The 60 n structure of the picornaviruses however does not allow definition of which the four non-identical polypeptide chains which comprise the pentamers and/or hexamers. More detailed experiments were needed to define the orientation of the polypeptide chains in the capsid of the virus.

Ruckert, et. al. (76,281) studied the dissociation product which resulted from interaction of purified cardioviruses with 0.1 M chloride ion at pH 5 to 6.5. The resulting product was shown by analytical ultracentrifugation and sodium dodecyl sulfate-polyacrylamide gel electrophoretic analysis to be a trimer, containing equal molar quantities of the α, β, γ, polypeptides. The trimer had a sedimentation coefficient of 13 to 14 S. The smallest polypeptide, δ, was found to precipitate with the virion RNA during the dissociation process. By further dissociating the trimer with 2 M urea it was found that a 5 S moiety was produced which also contained equal proportions of α, β, and γ polypeptides. These data suggested that the fundamental structural unit comprises one molecule of each of

the γ, β and α polypeptides joined together by hydrophobic interactions into pentamers. Each of these pentamers is centered at each of the 12 vertices of the icosahedral capsid. Thus the original 14 S structure observed during primary dissociation at pH 5 to 6 in the presence of 0.1 molar chloride ion is composed of 5 protomers of the 5 S fundamental structural units containing 1 molecule each of the α, β and γ species. The asymetric structural unit predicted by Finch and Klug (86) consists of 3 polypeptides, has a molecular diameter of about 68 Å, which is in agreement with the original prediction of Crick and Watson (58), and is repeated 60 times in the virus capsid.

Further insight into the orientation of the polypeptide chains has been obtained through a variety of biochemical analyses. Initially, VP 4 (δ) was believed to be a major constituent of the external surface of the virion. This conclusion was based on analysis of virions which were eluted from cells after virus adsorbtion. It was found that such eluted particles lacked the smallest capsid protein, VP 4 (δ) and were unable to reattach to cells. The RNA of these particles was intact and retained infectivity (59,178). Additional experiments in which virions were treated *in vitro* with acid, alkali, or urea produced non-infectious viruses which lost VP4(δ) and were antigenically similar to the eluted particles described before. Furthermore these chemically treated virions were shown to have the

same antigenicity as empty capsids normally produced during infection (143,154,180). These observations suggested the following: 1) During the normal course of infection two types of particles are normally produced. The first is infectious virus which has a density of approximately 1.34 g/cm^3 in cesium chloride solution (dense) and contains the full complement of the four nonidentical polypeptides species which confer D-antigenicity (D-antigenicity is identical to N or native antigenicity). The second particle is antigenically distinct (C-antigenicity) from the dense particles, did not contain RNA (coreless) and had a density of 1.30 g/cm^3 in cesium chloride solution. 2) The conversion from D to C antigenicity was accompanied by a loss of VP 4 (δ) when virus was treated in vitro with various chemical reagents or following attachment to susceptible cells. 3) That VP 4 (δ) confer upon viruses native, D antigenicity. 4) VP 4 is the protein which allows viruses to attach to cellular receptors (26,249).

In 1971 Mandel (191) reported that type 1 poliovirus could be resolved into two interconvertible components with isoelectric points of 7 and 4.5. Both components were infective. By comparing the two iso-electric components of polio type 1 virus to viruses bearing C-reactive antigens or D-reactive antigens, Mandel was able to conclude that the acidic component bore C-antigenicity and the alkaline component, D antigenicity. Similar studies were performed with human rhinoviruses and similar results were

found. Both of the isoelectric forms contained intact RNA and the full complement of capsid polypeptides (156). Indicated by these studies was that the apparent loss of VP 4, as measured by the conversion from D- to C- antigenicity was a consequence of, rather than the cause of inactivation.

The resolution of these data came from experiments in which intact virus, eluted particles, naturally occurring empty capsids, and disrupted (formerly infectious) virions were subjected to radiolabeling in vitro with a variety of reagents. These studies indicate that most picornaviruses appear to have VP 1 as the major capsid protein as assessed by reaction with iodine (39,179), N-succinimidyl propionate (343) and acetic anhydride (179). Moreover, when intact foot-and-mouth disease virions (a member of the genus Aphtovirus) were treated with trypsin or chymotrypsin, VP 1 polypeptides were cleaved resulting in a thousand fold loss of infectivity. Enzymatically treated virus was rendered incapable of eliciting production of neutralizing antibodies in guinea pigs (33,43,275). The VP 1 polypeptide of foot-and-mouth disease virus has also been shown to be a major constituent of the surface of the virus by radiolabeling studies of intact virions (286). Similar radiolabeling studies have been performed with mengo virus (a member of the genus Cardiovirus) and it was found that the α protein comprised much of the external surface. Tests with specific antisera demonstrated that only anti-α antibodies could block attachment of virions to cells (182). Thus it

appears that the major surface protein of picornaviruses is the VP 1 polypeptide and that the D to C antigenic conversion which occurs when virions interact with susceptible host cell receptors involves a major confirmational change in the capsid and may result in a loss of the VP 4 (δ) polypeptide.

Up to the present, studies designed to demonstrate the orientation of Theiler's virus capsid polypeptides have not been performed. A portion of this report will demonstrate that some of Theiler's virus capsid polypeptides have a surface orientation.

Studies of the replication of picornaviruses have added greatly to mans understanding of cell biology, the effects of viruses on host cells, as well as enabling investigators develop fundamental principles and techniques to define gene localization on a molecular level. Unlike the negative stranded RNA viruses, the picornaviruses do not contain a polymerase or replicase for synthesis of complementary nucleic acid. Because the viral genome has the same 5' to 3' polarity as cellular mRNA, it must be translated following unencapsidation to produce its replicase. Following translation, newly synthesized complementary RNA (cRNA) transcripts are synthesized by the replicase. These cRNAs in turn serve as templates for production of progeny vRNA and viral mRNA. These transcription events occur in complexes known as replicative intermediates. There are two forms of replicative intermediates (RI); the plus form which

consists of essentially a minus RNA (cRNA) template serving for transcription of several plus strands and the minus RI consisting of a plus strand serving as template for transcription of several minus strands. The RIs are extensively base paired(19,164). Replication could occur by either a conservative (plus and minus strands fully complexed) or semiconservative (one full plus or minus strand giving rise to minus or plus strands, respectively) mechanisms. By analysis of the RIs with regard to their stability in denaturants and RNAse susceptibility, it appears that the conservative mechanisms is the most likely form for the RI (19,164). Because the ratio of plus to minus strands produced during the course of the infectious cycle is maintained at about 10:1 (164), some regulatory mechanism for transcription must exist. Some of the plus strands will become covalently linked to the VPg protein at the 5' -end, encapsidated, and released from the cell during lysis. Other plus strands will serve as mRNA for the production of viral structural and non-structural proteins. Polypeptide 7c of poliovirus or p22 of EMC virus have been implicated to serve such a regulatory function (236).

One of the notable features of picornaviral infection is the virus' ability to nearly completely shut down host protein synthesis shortly after infection (65). Cellular rRNA and mRNA are both inhibited in cells infected with picornaviruses (64). The mechanism of inhibition of cellular protein synthesis following infection with polio-

virus is apparently due at least in part to viral specific inactivation of initiation factor eIF-4B (271). This factor is required for recognition of m^7G-capped cellular mRNA during polyribosome formation.

Because infection results in a near complete ablation of host protein synthesis it was possible to follow the appearance of viral specific polypeptides in infected cells during the course of infection. These experiments involved feeding radiolabeled amino acids to infected cells after viral induced host protein synthesis shut-down had been achieved, resulting in selective labeling of viral proteins. Summers, et. al., (313,315) identified approximately 14 different virus specific polypeptides in lysates of poliovirus infected cells. These polypeptides were separated one from another by sodium dodecyl sulfate - polyacrylamide gel electrophoresis. This procedure provided complete separation of these polypeptides and allowed good estimation of their molecular weights by comparing their relative mobility to the mobility of proteins of known molecular weights subjected to the same procedure. The total combined molecular weights of the proteins observed in acrylamide gels was approximately 500,000 daltons. This combined molecular weight exceeded the known coding capacity of the viral genome.

The estimated coding capacity of a viral genome can be determined from the molecular weight of the genome assuming that the triplet nucleotide code proposed by Watson and

Crick is non overlapping. For example, the genome of poliovirus has a molecular weight of about 2.6×10^6 daltons. The average mass of a nucleotide (as the sodium salt) is 344 daltons. Therefore the number of nucleotides in the poliovirus genome can be calculated by dividing the molecular weight of the genome by the average mass of a nucleotide arriving at a figure of 7558 nucleotides. The number of triplets coding for each amino acid can be determined by dividing the number of nucleotides by 3 yielding a total triplet coding capacity of 2519. Assuming an average mass for one amino acid to be 120 daltons (following removal of one molecule of water for each peptide bond) the maximum molecular weight of protein the genome could code for is equivalent to 302,320 daltons ((number of triplets) X (average mass of one amino acid)). The discrepancy between the theoretical coding capacity (302,320 daltons) and the accumulated mass of viral specific proteins observed by Summers, et. al. (500,000 daltons) was ultimately explained to be due to post-translational, viral protein processing.

Jacobson and Baltimore re-examined the translation of poliovirus RNA (128,129). These investigators found that when poliovirus specific polypeptides were synthesized in the presence of amino acid analogs (canavanine, p-fluorophenylalanine, azetidine-2-carboxcyllic acid) one large polypeptide was produced. The molecular weight of this polypeptide was larger than any polypeptide previously observed and represented the entire coding capacity of the

genome. Secondly, experiments in which protease inhibitors (diiospropyl fluorophosphate) were included and amino acid analogs were excluded, resulted in partial inhibition of proteolytic cleavage of the large polypeptide. When amino acid analogs and diiospropyl fluorophosphate were excluded, none of the large polypeptide was detected (128,129). These studies suggested that initiation of the translation of viral RNA occurred at a single site presumably near the 5' - end, and proteolytic cleavage of the largest polypeptide occurred before it was completely translated. Furthermore, analysis of tryptic hydrolysates of viral specific polypeptides indicated that all of the capsid proteins appeared to be cleavage products of one large precursor. This suggested that the mechanism of morphogenesis of viral polypeptides involved post-translational proteolytic processing of precursor polypeptide chains (128).

Further evidence to support this concept came from pulse-chase experiments in which virus infected cell cultures were pulsed for a short time with a radiolabeled amino acid mixture and then chased with excess unlabeled amino acids for varying lengths of time. It was found that the first viral specific product made was indeed a high molecular weight polypeptide. As the chase time increased the percent of label detected in viral specific polypeptide shifted from the high molecular weight polypeptide to lower molecular weight polypeptides. After very long chase periods most of the label could be detected in the capsid

proteins (121,129,313). Similar results have been obtained with enteroviruses, cardioviruses, rhinoviruses and foot-and-mouth disease virus (164,279). Pulse-chase studies offer the additional benefit of allowing determination of the half lives of each of the viral polypeptides as they are being processed from one larger precursor to their ultimate cleavage products (181).

With the knowledge that initiation of protein synthesis probably began at a unique site near the 5' -end of viral mRNA and resulted in the formation of one large polypeptide, it was possible to determine the gene order relative to the 5' - initiation site. The gene order was determined by using pactamycin, an inhibitor of chain initiation (48). When the drug is added to cells shortly after infection and pulsed with radiolabeled amino acids those proteins which have already been initiated will be translated to completion but no new chains can be initiated. Since proteins are synthesized from the amino- to the carboxyl-termini (5' to 3' direction on the mRNA) the carboxyl-end of the protein would have the highest radioactivity while the amino-end would have the least. Thus, the fraction of the total radioactivity incorporated into a viral specific polypeptide during translation in the presence of pactamycin relative to the fraction incorporated into the same polypeptide in the absence of the drug is a direct measure of the relative distance of that polypeptide along the mRNA from the 5' to 3'-end. In this manner the poliovirus gene order was de-

termined (36, 214, 240, 314, 316).

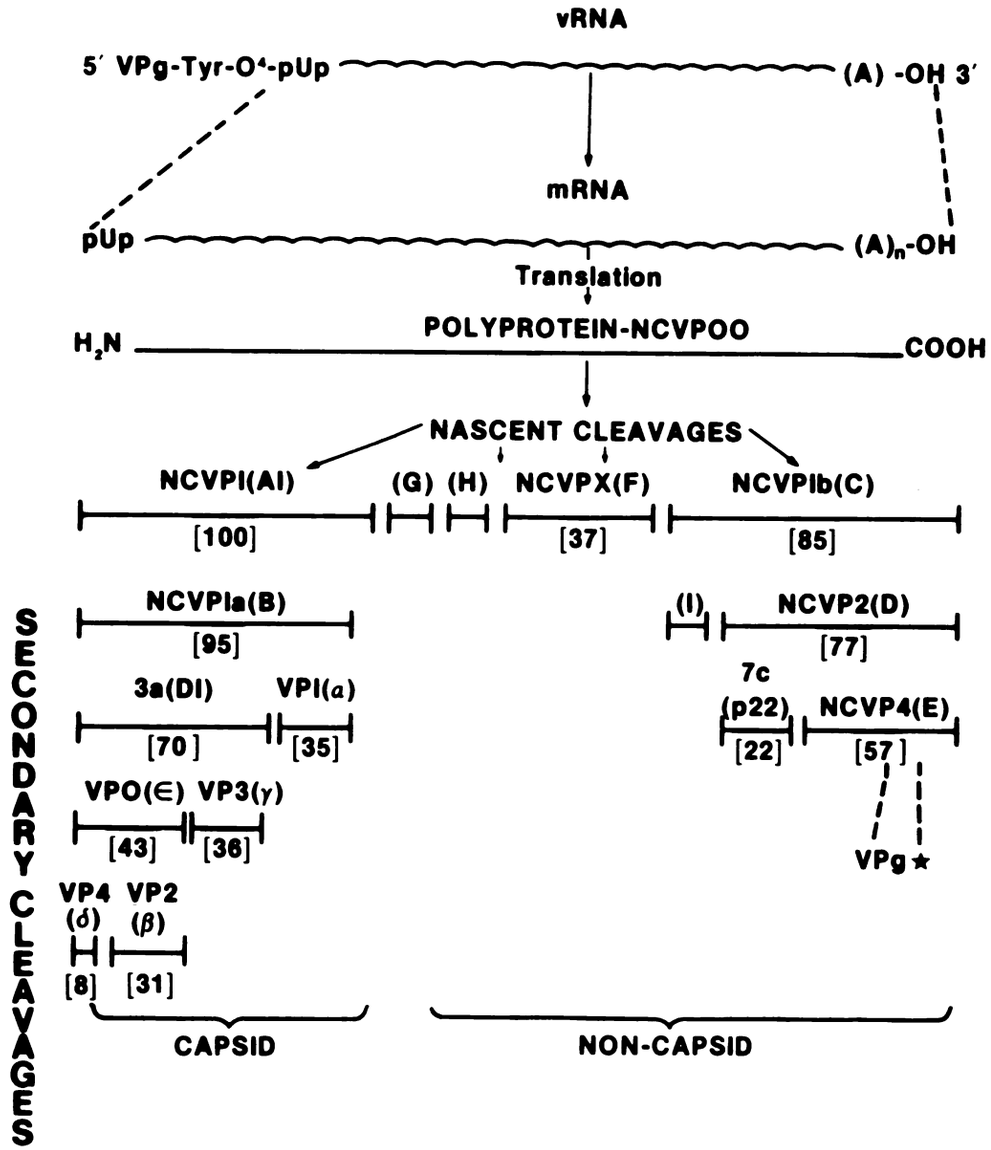
By coupling the pactamycin map, pulse-chase data, the precursor-product relationship determined by amino acid analog incorporation studies, tryptic peptide and cyanogen bromide mapping (34,70,128) it became possible to construct a scheme for the cleavage of picornaviral polypeptides and their orientation relative to the genome. An example is shown in Figure 1. It should be noted that there is little dispute about the gene order and function of the polypeptides derived from the 5'-end of the genome. However, functions of the polypeptides derived from the 3'-end of the picornavirus genome are currently being determined. Evidence indicates that the 3'-end of the poliovirus genome codes for the RNA-dependent RNA polymerase (replicase) of the virus founf in association with the replicative intermediate (9,9a,96,220). Poliovirus polyuridylic acid polymerase, and the RNA-dependent RNA polymerase have been isolated from infected cells and appear to be one in the same viral polypeptide (NCVP4 or p63) (87,335). This polypeptide maps near the 3'-end of the genome (see Figure 1). New evidence indicates that host factors may also play a role in viral RNA replication (66). Other products associated with the 3'-end of the viral genome include a protease (p22) which may also be involved in cleavage of the VPg protein from the 5'-end of RNA (236) (Fig. 1). Indeed recent reports

Legend to Figure 1

TRANSLATIONAL AND PROCESSING MAP OF PICORNAVIRAL PROTEIN.

Picornaviral RNA has a protein covalently linked to the 5' terminal pUp of the genome (VPg). mRNA lacks the VPg protein. Following translation the primary translation product, polyprotein-NCVP00 is nascently cleaved. Secondary cleavages ensue, and give rise to the various capsid and non-capsid polypeptides. The capsid polypeptides comprise structural components of the mature virion, the non-capsid polypeptides are mainly enzymatic in nature; the RNA-dependent RNA polymerase is coded for by the 3'-end of the genome. Nomenclature for the viral polypeptides correspond to the nomenclature for poliovirus (184,282), and the nomenclature in parentheses refer to the polypeptides of EMC virus (282). Map locations relative to the genome (vRNA) were determined by pactamycin mapping, pulse-chase, amino acid analog incorporation, tryptic peptide, and cyanogen bromide mapping studies (see text). The map location of VPg is indicated by the star (147,235)(see text). Numbers in brackets indicate the average molecular weight of polypeptides in kilodaltons.

FIGURE 1


vRNA
5′ VPg-Tyr-O⁴-pUp
(A) -OH 3′
mRNA
pUp
(A)n-OH
Translation
POLYPROTEIN-NCVPOO
H2N
COOH
NASCENT CLEAVAGES
NCVPI(AI)
(G) (H)
NCVPX(F)
NCVPIb(C)
[100]
[37]
[85]
SECONDARY CLEAVAGES
NCVPIa(B)
[95]
(I)
NCVP2(D)
[77]
7c
(p22)
NCVP4(E)
[22]
[57]
3a(DI)
VPI(a)
[70]
[35]
VPO(∈)
VP3(γ)
[43]
[36]
VPg★
VP4
(δ)
VP2
(β)
[8]
[31]
CAPSID
NON-CAPSID

indicate that VPg is a virus coded protein and is derived by proteolytic cleavage from one protein previously shown to have replicase activity (Figure 1) (235, 147).

Post-translational processing of viral polypeptides is clearly the mechanism by which ultimate viral products are made. The question remains, however, as to the origin of the processing enzyme(s). Without special inhibitory treatment such as the addition of amino acid analogs or protease inhibitors, three primary products are made during translation of the initial polyprotein. The *in statu nascendi* cleavages are thought to be due to cleavage signals built into the primary sequence of the polyprotein (153). Further processing of the nascent polypeptides appears to be performed by cellular proteases in association with membrane bound polyribosomes. By the use of cell free protein synthesizing systems employing picornavirus RNA as a source of messenger, primary and secondary cleavage products can be produced (35). The protease at least initially responsible for cleavage of the nascent polypeptide is associated with polyribosomes and not with 80 S ribosomes (153).

Cellular proteases are not solely responsible for proteolytic processing of viral proteins, however. Virus infected cells contain protease activity in greater excess

than uninfected cell and furthermore, if viral precursors are mixed with extracts of infected and uninfected cells, the extracts of only the infected cells are capable of carrying out proper cleavages of the viral precursors leading to the production of capsid polypeptides (152). Further evidence to support the existence of viral specific proteases has come from use of the reticulocyte cell-free protein synthesizing system which faithfully translated encephalomyocarditis viral RNA and produced a proteolytic activity. The protease produced in the cell-free system cleaved the coat precursor polypeptide of the virus into polypeptides which matched the molecular weights of virus capsid proteins produced in infected cells (244). Lawrence and Thach also reported discovery of a viral specific protein which was responsible for at least one capsid precursor cleavage event (160). These data indicate that at least part of the post-translational processing of picornaviral polypeptides is viral directed. Which of the viral polypeptides synthesized in infected cell is responsible for proteolytic cleavage(s) is still unclear. Preparations of purified picornaviruses were once demonstrated to contain proteolytic activity (120), but the possibility existed in these studies that a host enzyme may have contributed to the apparent proteolysis observed. Palmenberg, et. al. demonstrated that polypeptide p22 bears a protease activity responsible for cleaving capsid precursors. It was to be associated with, but not identical to, the RNA polymerase

(236). By comparing tryptic peptide digests of precursors and products of the replicase genes and the protease genes, these authors were able to demonstrate that the genes coding for the viral protease lie roughly 3' of the center of the viral genome, 3'- to the genes coding for capsid proteins and 5'- to the genes coding for the replicase (see Figure 1).

The ultimate production of mature virions is dependent upon replication and synthesis of progeny RNA molecules occurring in harmony with the proteolytic cleavage and synthesis of capsid proteins. The sequence of events during morphogenesis has been deduced from work with many picornaviruses, including encephalomyocarditis virus, poliovirus and foot-and-mouth disease virus (84,130,184,207,275). Following nascent cleavages of the polyprotein translated from viral RNA, the process of morphogenesis begins. The first step in morphogenesis involves aggregation of the 5 S precursor of polypeptides VP 0, VP 3 and VP 1 into a 14 S pentamer. Aggregation into the 14 S pentamer results in the cleavage of VP 0, VP 1 and VP 3 from each other. Twelve 14 S pentameric aggregates join together to form the procapsid ((VP 0, VP 1, VP 3)$_5$ 12) which has a sedimentation coefficient of about 75 S. Viral RNA (+ RNA with VPg attached to the 5'-end) appears to be inserted into the procapsid (84) resulting in the formation of the provirion which has a sedimentation coefficient of approximately 125-150 S.

Shortly after or conconmittant with insertion of RNA into the procapsid and formation of the provirion, the VP 0 protein is cleaved into VP 2 and VP 4 resulting in formation of the intact virion which has a sedimentation coefficient of about 150-156 S.

Preparation of purified virus from tissue culture supernatant or lysates of infected cells has been used to determine the steps in morphogenesis and to study the biochemical and antigenic properties of the sub-viral particles (248). These paricles and their properties are summarized in Table 7. Rate-zonal sucrose density gradient centrifugation of infected cell lysates revealed the presence of virion procapsids and procapsid precursors (14 S, 5 S). Infectious virions always exhibit D-antigenicity and the 5 S and 14 S pentameric procapsid precursors always exhibit C-antigenicity. Procapsids, differing in sedimentation velocity in sucrose gradients and bouyant density in cesium chloride gradients due to the absence of RNA, may exhibit either D or C-antigenicity, depending on the species of picornavirus (cf. Ruckert, 1976, ref. 276). Procapsids are equivalent to natural top component (NTC). When purified virus preparations are centrifuged in solutions of cesium chloride, two bands of viral particles may be observed. Virions, banding at a bouyant density of approximately 1.34 grams/cm^3, have the full complement of viral proteins, contain RNA, and bear D-antigenicity. A less dense band, having a bouyant density of 1.30 grams/ cm^3 may be observed. This less dense band, operationally referred to as arti-

TABLE 7

PROPERTIES OF PICORNAVIRUSES AND RELATED PARTICLES
ISOLATED FROM INFECTED TISSUE CULTURE FLUIDS OR CELL LYSATES[a]

PARTICLES	COMPOSITION	RNA	SEDIMENTATION PROPERTIES	ABILITY TO ATTACH TO CELLS	ANTIGENICITY
Virion	VP1,2,3,4	+	1.35 gm/cm^3[b] ~156 S[c]	+	D
Procapsid	VP0, 1, 3	-	1.30 gm/cm^3 ~ 75 S	+/-[d]	D/C[d]
14 S Precursor	VP0, 1, 3	-	14 S	-	C
5 S Precursor	VP0, 1, 3	-	5 S	-	C
Artificial Top Component (ATC)	VP1, 2, 3	-	1.30 gm/cm^3	-	C
Natural Top Component (NTC)	VP0, 1, 3	-	1.30 gm/cm^3	+/-[d]	D/C[d]
Acidic Isoelectric Form (pI=4.5)	VP1,2,3,4	+	1.34 gm/cm^3 ~140 S	-	C
Basic Isoelectric Form (pI=7.0)	VP1,2,3,4	+	1.34 gm/cm^3 ~150 S	+	D

[a] Compiled from references 156, 184, 191, 295, and 279.

[b] Bouyant density in cesium solutions (CsCl and Cs_2SO_4).

[c] Sedimentation coefficients in rate-zonal sucrose gradients ($S_{20\omega}$).

[d] Some preparations of procapsids or NTC have only one antigenic configuration while others have mixtures of D and C. No individual mosaic particles bearing both D and C antigenicities have been observed (cf. Rueckert, ref. 279).

ficial top component (ATC), contains particles which bear polypeptides VP1, 2, and 3 and are C-antigenic. The less dense band may also contain particles of NTC but these may be distinguished from ATC by their D-antigenicity, however, this is not always possible depending on the type of picornavirus under investigation. The NTC particles of some picornaviruses may exhibit either D- or C-antigenicity and therefore, by this criterion alone, it is impossible to distinguish NTC particles from ATC particles. NTC particles have a different polypeptide composition than ATC particles, however. NTC particles have VP 0, 1 and 3, whereas ATC particles have VP 1, 2 and 3. If care is taken during the preparation of the cell lysate or purified virus preparation prior to cesium chloride gradient centrifugation, procapsids (NTC particles) can be separated from virions and therefore during centrifugation of the virus preparation any particles observed banding at a density of 1.30 grams/cm^3 will more likely represent ATC particles.

Thus, it appears that C-antigenicity (ATC particles) correlates with particles containing VP1, 2 and 3 and an inability to attach to cells. D-antigenicity (virions, procapsids and NTC particles) correlates with a positive ability to attach to cells. NTC particles (procapsids) can be either D- or C-reactive, do not contain RNA, and bear VP 0, 1 and 3, whereas virions contain RNA and bear VP 1, 2, 3 and 4, and are D-reactive.

The antigenic characteristics and morphogenesis of Theiler's virus have not been reported. The only report detailing a polypeptide composition of Theiler's virus was by Lipton and Friedmann (176). Furthermore, the gene order of the Theiler's viruses remains undescribed; however, because the Theiler's viruses are members of the Picornaviridae, the gene order is probably very similar to the other members of the genus. Although it is known that the genome of the Theiler's viruses is RNA (89), no reports until the present one have been made describing its molecular weight or sedimentation velocity. Indeed, Theiler's viruses have been the object of pathologic and pathogenic studies rather than molecular virologic ones. It would not be surprising to discover that Theiler's virus shares many of the antigenic characteristics (conversion from D- to C-antigenicity upon heating or treatment with chemical reagents; presence of multiple pI forms of the virus) with other members of the Picornaviridae. It is also likely that the poly-protein cleavage-assembly scheme of morphogenesis common to the other picornaviruses will also be the case for Theiler's virus.

Interest in studying the molecular virology of Theiler's virus, although still in its infancy, stems from the fact that many of the strains of this group of murine viruses have the potential to induce central nervous system demyelination concomitant with a low level of viral persis-

tence. It is hoped that study of the molecular biology of viruses will provide the foundation from which the mechanism(s) of viral persistence can be elucidated.

Mechanisms of Viral Persistence and Production of Chronic Disease:

Several viruses from a wide number of genera have been shown to have an ability to persist in a susceptible host following either natural or experimental infection. Persistent viral infections can be characterized by either a low or high level of cell-free virus production, or an absence of cell-free virus production but with maintenance of either viral genome equivalents within infected cells or limited expression of viral antigen(s). The spectrum of viral persistence may be present with or without production of obvious clinical disease of the host.

An example of a persistent viral infection of humans which is characterized by a high level of virus expression concomitant with chronic progressive disease is progressive multifocal leukoencephalopathy (PML) (13,136, 321). Examples of persistent viral infections characterized by low or no expression of cell-free virus resulting in chronic progressive disease are progressive rubella panencephalitis (PRP), and subacute sclerosing panencephalitis (SSPE). These three disease are caused by conventional viruses; that is viruses which have been shown to have generally typical structure, physio-chemical and biological characteristics. Careful analysis of viruses recovered

during these diseases has revealed that subtle differences do exist between persistent viruses and their wild type, or presumed wild type parents. Two progressive chronic disorders of man caused by unconventional agents; i.e. agents which have not been visualized, isolated or well characterized biologically or physio-chemically, include Kuru and Creutzfeldt-Jakob disease (CJD) (13,92,135,136,321).

Other viruses have demonstrated to persist in vivo, express little or no viral antigen in those organs or tissues harboring the virus, and cause only episodic periods of acute illness. Examples of viruses which cause these persistent infections in man include Herpes Simplex viruses and Varicella-Zoster virus.

Naturally occurring persistent infection due to conventional viruses in animals include Visna and Maedi in sheep, old-dog disease, a complication of canine distemper in dogs, lactate dehydrogenase-elevating virus (LDV), and lymphocytic choriomengitis virus (LCM) infections of mice, Aleution mink disease of mink, and infectious equine anemia in horses (105,192,201,257,302,321) in addition to Theiler's virus infection of mice (63,170,172,173,175,192,312,324, Stroop, results reported here). Two disease of animals shown to involve persistent infection by unconventional agents include Scrapie in sheep, and transmissible mink encephalopathy (TME).

Within each of the viral animal-model systems the mechanism of viral persistence are generally incompletely understood. Most acute viral infections are elminated from the host by a concerted production of antibody to the infecting agent and components of the cellular immune system. For a virus to persist following an acute phase of replication in an immunocompetent host requires that the virus be able to evade normal immune surveillance. Persistence could be accomplished in four general ways. The first is if the agent could maintain itself in a "protected site." These sites could be intracellular where the viral genetic information could be intranuclear, either as an integrated genome or as an episome, or intracytoplasmic, where the genome could be proteced from intracellular nucleases by being in association with viral specific nucleoprotein. Other "protected sites" could be in an organ system which is separated from antibody contained in the vascular system by an especially tight endothelial barrier, e.g. brain. Another "protected site" for viruses to persist and evade the immune system would be within cellular elements of the immune system itself. A second way by which a virus could persist is if the virus itself was non-immunogenic. In this case an agent could persist simply because the host would not mount an immune response to it. A third way viruses could persist is if during interaction with the host they were to elicit a large quantity of non-neutralizing antibody. This non-

neutralizing antibody could serve two functions. First, it could in effect serve as a blocking antibody protecting virus from any neutralizing antibody produced by the host. Secondly, the non-neutralizing antibody in association with the virus could be phagocytosed by the reticuloendothelial system perhaps allowing the virus to infect the cells of the reticuloendothelial system itself. A fourth general way by which a virus could persist is if the virus was capable of inducing tolerance within the host. In this instance the virus would have no need to evade immune surveilance for it would not be regarded as a "nonself antigen" within the tolerant host. If the virus was relatively noncytocidal, the virus would be able to replicate freely without impairment from the immune system. In an immunoincompetent host, either with respect to its ability to produce antibody or mount a cellular immune response to the infecting agent, ineffective viral clearance will result thus allowing the virus to persist at least until accumulative cytolytic damage results in death of the host.

The examples of the persistent viral infections of man and animals given above are representative of each of these mechanisms of establishment and maintenance of persistence; some of these infections will be examined in detail below. Figures 2, 3 and 4 diagramatically outline detailed theoretical mechanisms of viral persistence and induction of chronic disease by conventional and unconventional agents grown in permissive and nonpermissive cell types. A per-

missive cell is defined as one which allows for complete expression of viral genetically coded functions, and a nonpermissive cell type is defined as a cell which does not allow for complete viral expression, including lytic production of free virus, but rather results in lysogenous interaction, an abortive or otherwise incomplete expression of virally encoded functions.

Slow Virus Infections caused by Unconventional Agents:

Kuru, CJD, Scrapie and TME are among those unconventional agents which have been termed "slow virus" infections (299), with incubation periods measured in years; they produce chronic degenerative diseases in their respective hosts which always lead to death (see Figure 2) (92). These unconventional agents are capable of evading the immune system of the host because they are nonimmunogenic (92). The nonimmunogenicity of these agents can be inferred from the lack of an inflammatory response in the brain, absence of pleocytosis or elevated protein levels in cerebrospinal fluid, and from an ansence of a host immune response to the causative agent (92). Recently, Sotelo, Gibbs and Gajdusek have demonstrated autoantibodies in patients, with Kuru and CJD, directed against axonal neurofilaments (303). These studies documented the first evidence of an immune response associated with these agents, directed not against the agents themselves but rather against normal "self-antigens."These agents produce a progressive vacuolation in the dendritic

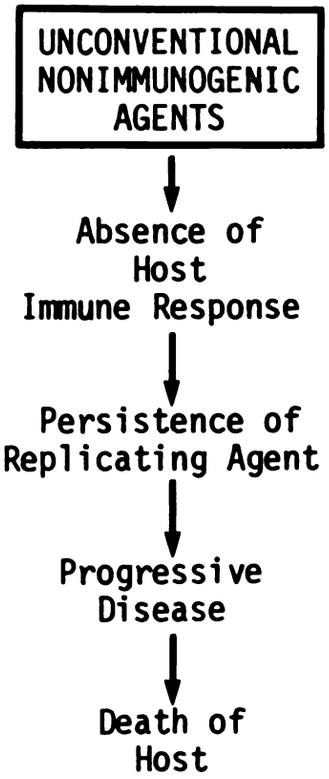


Figure 2

MECHANISM OF VIRAL PERSISTENCE AND PRODUCTION OF CHRONIC DISEASE DUE TO UNCONVENTIONAL AGENTS.

Unconventional agents are infectious agents which have not been visualized, isolated or well-characterized biologically or physiochemically. Diseases produced by such agents include Kuru and Creutzfeldt-Jakob disease in man and Scrapie and transmissible mink encephalopathy in animals. The unconventional agents have not been demonstrated to elicit an immune response in an infected host, hence can replicate and persist in vivo without impairment by host defense mechanisms. These diseases are progressive and uniformly fatal.

and axonal processes and cell bodies of neurons and occasionally in astrocytes and oligodendrocytes. The end stage of these disease process is status spongiosis of gray matter (13,92,136).

The remaining viral diseases of which viral persistence is a feature are caused by generally conventional immunogenic agents. Figures 3 and 4 illustrate the theoretical mechanisms by which these agents could persist in nonpermissive and permissive cell types. A few of these diseases will be discussed below to illustrate these mechanisms of viral persistence as they relate to disease production.

Progressive Multifocal Leukoencephalopathy (PML):

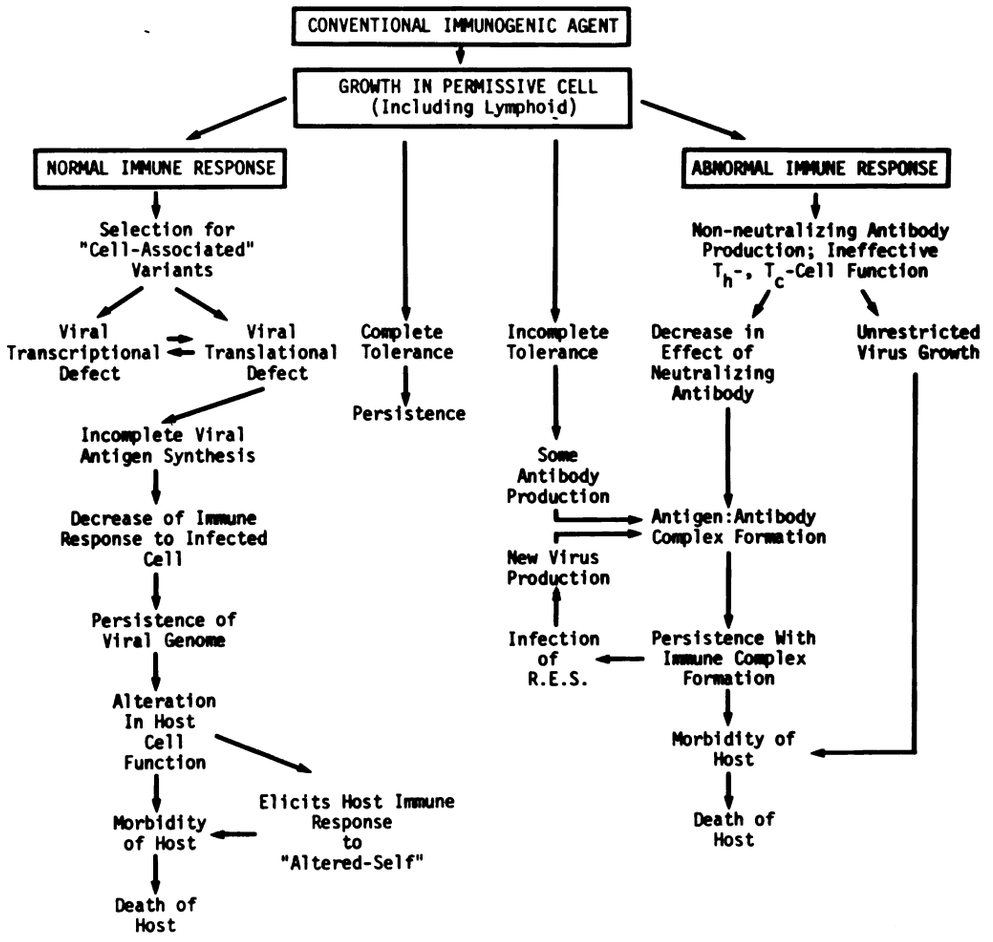
PML is an uncommon central nervous system disease of humans and is caused by an opportunistic papovavirus infection in patients with underlying disorders such as reticuloendothelial diseases, inflammatory diseases associated with a state of secondary immunodeficiency (139), Hodgkin's disease, lymphomas, leukemias, carcinoma and other malignancies, sarcoidosis, and in patients who have received immunosuppressive therapy (13,136). Patients with PML exhibit neurological signs such as paralysis, mental deterioration, visual loss, sensory abnormalities, and ataxia. The disease is progressive and usually leads to death in less than one year. Pathologic lesions are characterized by demyelination, enlarged oligodendrocytes, nuclei containing eosinophilic inclusions containing viral particles (356) and

Legend to Figure 3

MECHANISMS OF VIRAL PERSISTENCE AND PRODUCTION OF CHRONIC DISEASE DUE TO CONVENTIONAL IMMUNOGENIC AGENTS REPLICATING IN PERMISSIVE CELL TYPES.

The figure illustrates mechanisms of persistence due to agents which can produce complete or incomplete tolerance in their host and agents which induce normal or abnormal immune responses following infection. Examples of agents which may be capable of inducing complete or incomplete tolerance in their respective hosts include rubella virus in humans resulting in progressive rubella panencephalitis and lymphocytic choriomeningitis virus in mice of various ages (for details, see text). A disease in which the immune system has been postulated to select for "cell-associated" variants as a mechanism for viral persistence is subacute sclerosing panencephalitis (SSPE) (see text). Abnormal immune responsiveness or induction of incomplete tolerance in an infected host may lead to the production of non-neutralizing antibody which may result in immune complex disease. Examples of diseases which involve immune complex formation include progressive rubella panencephalitis, Aleutian mink disease, lymphocytic choriomeningitis, and Equine Infectious Anemia (for details, see text).

FIGURE 3


CONVENTIONAL IMMUNOGENIC AGENT
GROWTH IN PERMISSIVE CELL (Including Lymphoid)
NORMAL IMMUNE RESPONSE
ABNORMAL IMMUNE RESPONSE
Selection for "Cell-Associated" Variants
Viral Transcriptional Defect
Viral Translational Defect
Incomplete Viral Antigen Synthesis
Decrease of Immune Response to Infected Cell
Persistence of Viral Genome
Alteration In Host Cell Function
Morbidity of Host
Death of Host
Elicits Host Immune Response to "Altered-Self"
Complete Tolerance
Persistence
Incomplete Tolerance
Some Antibody Production
New Virus Production
Infection of R.E.S.
Non-neutralizing Antibody Production; Ineffective T_h-, T_c-Cell Function
Decrease in Effect of Neutralizing Antibody
Unrestricted Virus Growth
Antigen:Antibody Complex Formation
Persistence With Immune Complex Formation
Morbidity of Host
Death of Host

large, bizarre dysplastic astrocytes, but little or no inflammatory response (13).

Two papopaviruses, JC virus and SV_{40}-PML virus (233, 340) have been recovered from PML brain material. JC and SV_{40}-PML viruses appear to be highly cell-associated requiring fusion of explant cultures of PML brain with primary monkey kidney cells to recover infectious virus (321). Ultrastructural studies have shown that papovavirus particles are frequently found in oligodendroglial cells and rarely in astrocytes; immunofluorescence studies of papovavirus antigen distribution in brain lesions indicate more oligodendroglial cells bearing papovavirus T-antigens than astrocytes (321). Oligodendroglial cells therefore probably represent a permissive cell type which allows virus replication and is therefore lysed by the virus (Fig. 3). Individuals who are immunosuppressed either through iatrogenic manipulation or a malignant disease process, and become infected with JC or SV_{40}-PML viruses, are susceptible to a lytic infection of oligodendroglia (Fig. 3). Serologic studies indicate that by 14 years of age, 65% of individuals have acquired antibody against JC virus, and at a later age, 72% of the population have humoral immunity to the agent (234), indicating a widespread subclinical infection. The virus, however, displays little virulence. Thus, it is only in those individuals who are immunosuppressed that the virus is capable of persisting and causing slow virus disease.

The hypertrophied astrocytes present in PML lesions suggest the possibility that the PML agents are capable of inducing transformation of these cell types. Transformation by the oncogenic DNA viruses and RNA tumor viruses usually occurs following infection of non-permissive cell types; infections of permissive cell types results in a lytic infectious process with release of viral progeny (18, 82, 336). Circumstantial evidence to support this postulate has been obtained using in situ hybridization to localize JC virus DNA in PML brain (74). These studies revealed a heavy label on oligodendroglial nuclei but only a small amount of label on the astrocytes within PML lesions. This data does not prove nor disprove the hypothesis of a non-permissive infection of astrocytes, but at least indicates that astrocytes can be infected by the virus and are not exclusively reacting to injury. If, however, a non-permissive infection of astrocytes does occur, then such an infection would allow for at least partial persistence of viral genetic information within astrocytes (see Figure 4). In this infection, the virus may persist as an integrated or episomal genome within the nuclei of astrocytes and result in partial synthesis of viral antigens. That immunofluoresence was at least partially observed in astrocytes supports this hypothesis.

Subacute Sclerosing Panencephalitis (SSPE):

SSPE is another persistent viral infection of humans. It is a chronic progressive encephalitis of childhood or

adolescence and is caused by measles virus (1,3,13,21,53, 67,101,132,133,136,246,289,320,321,322,334). The disease appears to be associated with natural measles infection occurring in individuals under the age of 2 years. The disease is characterized clinically by subacute development of behavioral changes, and difficulty with language. The disease progresses to development of myoclonic seizure activity and worsening dementia. Patients with SSPE become progressively obtunded, lose contact with the environment, become oblivious to their surroundings, lapse into coma, and eventually die.

Pathologically, SSPE brains are characterized by lesions distributed throughout most of the brain in both white and gray matter with the exception of the cerebellum. The lesions themselves may be necrotic, exhibit primary and secondary demyelination and astrocytosis. An inflammatory reaction is usually present consisting of lymphocytes and plasma cells. Eosinophilic nuclear inclusions are present, and are pathognomonic for SSPE. Occasionally cytoplasmic inclusions may also be seen in neurons, oligodendroglia and astrocytes (13,136). The inclusions are composed of paramyxovirus-nucleocapsids; immunofluorescent staining of brain sections indicates the present of measles antigens in both the nucleus and cytoplasm.

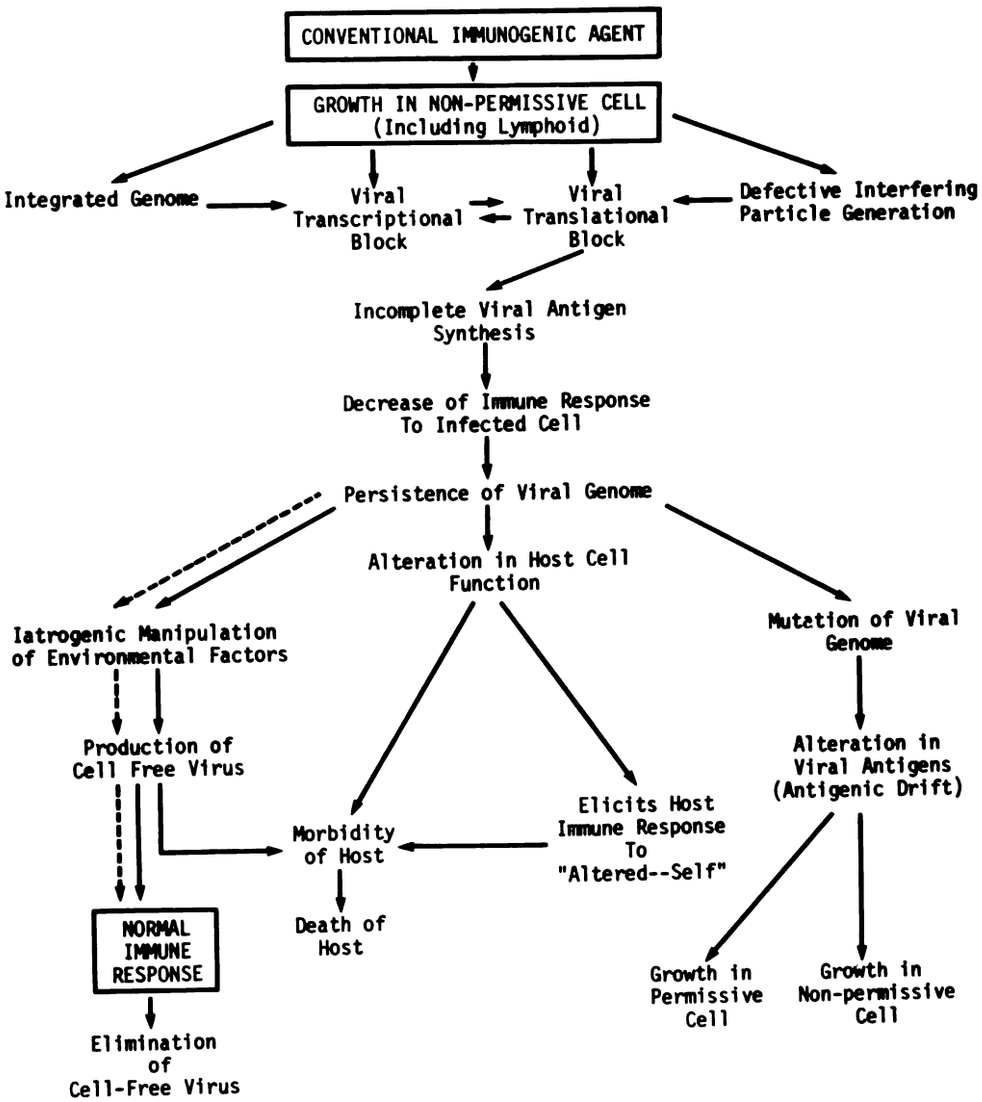
The host-virus relationship in SSPE is characteristic of virus grown in a non-permissive cell. This is suggested from the observation that conventional virus isolation

Legend to Figure 4

MECHANISMS OF VIRAL PERSISTENCE AND PRODUCTION OF CHRONIC DISEASE DUE TO GROWTH OF CONVENTIONAL IMMUNOGENIC AGENTS IN NON-PERMISSIVE CELL TYPES.

The figure illustrates possible mechanisms by which retroviruses and other viruses capable of integration or episome formation may persist and produce morbidity of the infected host. The dashed line indicates the mechanism of reactivation of a persistent viral genome (eg. Herpes Simplex Virus reactivation) due to iatrogenic manipulation or environmental factors (see text for details).

FIGURE 4


CONVENTIONAL IMMUNOGENIC AGENT
GROWTH IN NON-PERMISSIVE CELL (Including Lymphoid)
Integrated Genome
Viral Transcriptional Block
Viral Translational Block
Defective Interfering Particle Generation
Incomplete Viral Antigen Synthesis
Decrease of Immune Response To Infected Cell
Persistence of Viral Genome
Alteration in Host Cell Function
Iatrogenic Manipulation of Environmental Factors
Mutation of Viral Genome
Production of Cell Free Virus
Alteration in Viral Antigens (Antigenic Drift)
Morbidity of Host
Elicits Host Immune Response To "Altered--Self"
NORMAL IMMUNE RESPONSE
Death of Host
Growth in Permissive Cell
Growth in Non-permissive Cell
Elimination of Cell-Free Virus

techniques have always failed to rescue the causative agent from SSPE brain material. Co-cultivation of trypsinized brain cells with tissue culture cells permissive to measles virus is required to isolate the agent (243). Those isolates which have been recovered from brain have been shown to differ in certain biologic and morphologic characteristics, but share antigens common to wild type measles virus (136). SSPE isolates are defective in that they most often fail to produce complete extracellular virus. An animal model of SSPE which results in persistent CNS measles infection of hamsters has been developed (37,134). This model has indicated that the host antibody response to measles virus seems to be involved in converting the virus from a cell-free to a cell-associated persistent state. A recent report has indicated that multiple variants of measles may be present within virus stock used for pathogenetic studies (38).

Two theoretical mechanisms can be put forth to explain the persistence of measles virus in SSPE. The first is that an individual is infected with a population of variants of measles virus, sharing many antigenic similarities with each other and with natural measles virus. This population undergoes a selection process due to the normal immune response of the host (Figure 3). The immune response selects for those "cell-associated" variants which have either transcriptional and/or translational defects resulting in

incomplete viral antigen synthesis. Recent evidence by Johnson, et. al. (137) indicates that one measles virus virus antigen (matrix (M) protein) selectively disappears during the course of infection in experimental SSPE in the hamster, at the time when serum antibody levels are increasing. Incomplete viral antigen synthesis could provide for a decrease in antigen epitope density on the surface of an infected cell and thereby allow the cell to evade immune killing by cytotoxic T cells (T_c), antibody directed cell cytotoxic mechanisms (ADCC), or natural killer-cell (NK) mechanisms. This would allow for persistence of the viral genome (Fig. 3). The second mechanism involves infection with a homogenous population of conventional measles virus. Because SSPE agents usually require sophisticated virologic procedures to rescue them from diseased brain, it is conceivable that the virus is present and replicating in a non-permissive cell type (Fig. 4). In this case, the non-permissive cell type would allow for incomplete viral antigen synthesis by specific or non-specific factors disrupting normal transcription of viral nucleic acid or translation of their gene products. Perhaps a young host (less than two years of age) contains a higher number of non-permissive cell types accounting for the age distribution of SSPE.

Either mechanism would result in incomplete viral antigen synthesis, a decrease of the immune response to the infected cell, and thus ensure persistence of the viral genome (Figure 3 and 4). Clearly both mechanisms may be

operative in SSPE. The persistence of the viral genome and limited expression of viral antigens may well serve to alter normal host cell function. Alteration in host cell function may well result in cellular expression of cellular antigens which are normally sequestered from the immune system. Sequestered antigens, perhaps in association with minimal amounts of viral antigens may be viewed as nonself by the immune system and elicit an immune response to "altered-self" (Figures 3 and 4). Panitch, et. al. (238) have detected antibodies to myelin basic protein in the cerebrospinal fluid of patients with SSPE, which supports the hypothesis that an "autoimmune" mechanism may be involved in the pathogenesis of the disease. Alterations in host cell function plus an elicitation of an autoimmune response to cells harboring persistent viral genomes will eventually lead to destruction of the infected cell. Accumulative cellular damage will eventually lead to production of clinical disease, progressive morbidity, and death of the host (Figures 3 and 4).

Progressive Rubella Panencephalitis (PRP):

Another chronic central nervous system viral infection which has a clinical syndrome quite similar to SSPE is PRP (13,136). Rubella virus has been demonstrated to persist within the central nervous system and the lens of the eye into childhood after *in utero* infection during the first trimester of pregnancy (13,69,102,136,196). Recently,

rubella virus has been demonstrated to persist within the central nervous system and produce PRP after a long period of stability within the host (329). In most cases, PRP has occurred in children with definite stigmata of congenital rubella. PRP is an extremely rare disease; as of 1980 only 7 cases were described (31,116). Of these 7 cases, 4 developed the disease as an apparent sequelae to congenital rubella, two developed the disease with unknown primary exposure and one with early childhood rubella (131,329).

PRP is an inflammatory disease exhibiting perivascular cuffing with some invasion of the brain parenchyma with lymphocytes. Microglial nodules may also be observed. Perivascular deposits of an amorphous periodic acid Schiff-positive material are also present in PRP; this material is frequently observed in brains of children suffering from congenital rubella syndrome. There are no inclusions present in brain tissue (13,136). Cremer, et. al. were able to isolate rubella virus from brain biopsy material (57), and Wolinsky, et. al. recovered the virus from peripheral blood leukocytes (345). Individuals with PRP have remarkable increases in serum and CSF IgG, which is capable of producing an inhibition of the rubella hemagglutination assay in vitro (321); antibody to rubella virus is produced intrathecally within the central nervous system (291,339,344). In contrast with SSPE, the cerebellum is markedly degenerated and there appears to be a greater white matter involvement in PRP than

SSPE (330). Cell mediated and humoral immune responses have been examined and have been inconclusive in demonstrating a consistent defect which would explain the pathogenesis of PRP (57,131,291,321,339). In two cases, increasing serum levels of interferon were detected, and serum from these cases were shown to interfere with production of interferon by normal donor lymphocytes, in vitro (345).

Although it is probably premature to speculate on the persistence of rubella virus in PRP, available evidence does suggest that some degree of tolerance of the host to rubella virus may be involved and allow for persistence of the virus (Figure 3). Tolerance is identified as specific immunologic unresponsiveness to an immunogen, which normally induces an immune response in a syngeneic host. Rubella virus infection in utero could produce a certain degree of tolerance to the virus. Induction of tolerance to heterologous (non-self) proteins during neonatal life has been demonstrated experimentally by Habicht, et. al (107). The hypothetical tolerance induced in utero to rubella virus is supported by the observation that the virus is able to persist into childhood for considerable lengths of time in children with congenital rubella syndrome (12,196). If the tolerance to rubella virus is complete, that is, a complete inability of both T- and B- lymphocytes to respond to the virus, no immunoglobulin to viral antigens would be produced, and no cell-mediated immune response to viral antigens would

exist in such tolerant individuals (338). Because patients with congenital rubella and PRP do make IgG, and in most instances show no major defects in cellular immunity, the hypothetical tolerance induced in utero must be incomplete (Fig. 3). It is a matter of conjecture as to whether the proposed tolerance is due to partial clonal elimination of B-lymphocytes, the precursors of antibody secreting plasma cells, T-lymphocytes of the helper (T_H) class, or augmentation of the suppressor effects of suppressor T-lymphocyte (T_S) class (95). In any case, continued replication and release of progeny virus coupled with at least partial immune unresponsiveness to the agent would provide for a situation in which free virus, and viral antigen in association with cell membranes of lysed cells, (lysed by virus or immune mechanisms) could complex with antibody and circulate as antigen:antibody complexes (Fig. 3). These complexes, if of the appropriate size, could be removed from the circulation by the reticuloendothelial system, or other phagocytic cells (macrophages), and deposit in the glomeruli of the kidney.

Recent evidence has been obtained that immune complex formation does occur in the CSF and serum in 2 patients with PRP (55). These immune complexes contained rubella specific IgG antibody as assayed by radioimmunoassay following removal of the immune complexes from serum or CSF by absorption to Raji cells. The immune complexes were of various sizes ranging in density from 1.14 gm/cm^3 to 1.26

gm/cm^3. In the case of PRP, it is unclear whether reinfection of phagocytic cells occurs at all, and to what degree immune complex formation contributes to the pathogenesis of the disease. Evidence will be presented below with other animal-viral models of persistence to indicate that reinfection of the reticuloendothelial system of the host can play a major role in pathogenesis and maintenance of persistent viral infections.

Murine Lymphocytic Choriomeningitis Virus (LCM) Infection:

One of the best studied animal models in which tolerance of the host plays a crucial role in establishing a persistent viral infection with or without immune complex disease late in life, is murine lymphocytic choriomeningitis (LCM) virus infection of mice. LCM is a member of the Arenavirus group. Because this virus buds from infected cell membranes and is not overwhelmingly lytic, the pathology observed in LCM infection is immunopathologic, caused by the host's immune response.

The clinical manifestation of LCM infection in mice depends on the strains of virus and mice used, route of infection, virus dose and most interestingly, the immunocompetence of the host (49,72,352). Totally immunoincompetent mice (fetal mice in utero, newborn less than 24 hrs. old, nude mice, adult mice which have been thymectomized, lethally irradiated and bone marrow reconstituted, mice

treated with cyclophosphomide at the time of or soon after infection, or mice treated with anti-lymphocyte serum), infected with LCM by the intracranial or intravenous routes, survive and become carriers of LCM virus. These mice can be demonstrated to have a lack of virus specific cytotoxic T-lymphocytes, but produce non-neutralizing antibodies to LCM (201,352). Production of antibodies to the virus may lead to immune complex disease later in life. Suckling mice have a relatively greater degree of immunocompetence than fetal mice but less immunocompetence than adult mice. Intracranial or intravenous inoculation of suckling mice results in a mixture of clinical manifestations. Some mice may develop a "runting" syndrome, some mice will die from acute lymphocytic choriomeningitis, a few will recover from this disease, and still others will become chronic carries of LCM virus (352). When immunocompetent adults are infected with LCM virus by the intracranial route, all mice will die within 6 to 8 days after infection of acute lymphocytic choriomeningitis (352). After intravenous infection of the virus, adult mice being to die after 10 days post-infection (352).

It has been convincingly demonstrated that LCM virus infection of immunoincompetent mice results in cytotoxic T-lymphocyte tolerance (49,352). Figure 3 illustrates the mechanism of persistence of LCM virus in immunoincompetent mice. In this model, incomplete tolerance results in the

production of antibody, albeit at relatively low levels. Infected cells are not lysed by viral specific cytotoxic T-lymphocytes because of tolerance to the virus. Mice become carriers of LCM virus because the virus itself is not particularly lytic, thus allowing the animal to survive. Antigen:antibody complexes may form in the circulation of tolerant, infected mice and eventually deposit in the glomeruli of the kidney. Accumulative complex deposition results in complement fixation and eventual glomerulo-nephritis.

Interestingly, the LCM virus-mouse model provided the first demonstration that the specificity of cytotoxic T-lymphocytes is not for the virus alone, but also for the cell surface marker coded by the major histocompatibility complex (H-2) (353,354,355,356,357). Immune Tc-lymphocytes (cytotoxic T-lymphocytes) will only lyse an infected cell target if the Tc-lymphocytes and the infected cell share homologous H-2 K or D antigens. Only the K and D region genes, which code for the major transplantation antigens, need to be in common between target and effector cells; the I region genes of the major histocompatibility locus, which code for immune responsiveness and Ia cell-surface antigens, are not involved in specific virus-cell cytolytic interactions. H-2, K or D alleleic specificity of Tc-lymphocytes has been demonstrated for a variety of virus-cell systems in addition to LCM, including Sindbis, ectromelia, vaccinia,

influenza and Sendai viruses (5,50,73,94,119,157,206,355). Recently, the H-2 specificity restriction on cytolysis of infected cells by Tc-lymphocytes has been demonstrated during Coxsackie virus B-3 infection of myofibers in vivo and in vitro (346,347).

The H-2 restriction can be demonstrated in vivo if chronic carriers of LCM virus (induced by infection of immunoincompetent mice), are inoculated with syngeneic LCM primed Tc-lymphocytes. Recipient mice will die of acute lymphocytic choriomeningitis (49,72,352). If tolerant mice are inoculated with LCM primed, Tc-lymphocytes bearing histocompatibility antigens incompatible with their own major histocompatibility antigens, no death will occur (352).

Lactic Dehydrogenase Virus (LDV) Infection of Mice:

Another virus-animal model of persistence which is associated with late-onset glomerulonephritis is Lactic Dehydrogenase virus (LDV) infection of mice (230,259). In contrast to LCM virus, most strains of LDV produce a life-long asymptomatic infection when inoculated into mice of any age (203).

LDV infection in mice produces widespread lymphoid hyperplasia and splenomegaly (254). Interestingly, shortly after LDV infection, mice exhibit a transient decrease in the number of lymphocytes in the thymus, in the thymus-dependent areas of the spleen, and in the blood (300). LDV

is capable of replicating in macrophages in vitro (27,252) and in primary explants enriched for reticuloendothelial cells (79,251,350). Electron microscopy of peritoneal macropahges of LDV infected mice revealed virus particles within these cells (225).

As with LCM infection, there is a persistent viremia associated with LDV infection of mice. Infectious virus circulates as a complex with host IgG (203). Immune complexes have been examined using an indirect immunofluorescence test; anti-LDV antibody can readily be detected in these complexes (201,259).

Not all of the immunoglobulins produced during LDV infection are neutralizing (201,228,259). This is indicated in part by the observation of infectious virus:antibody complexes present in the serum of LDV infected mice (259); serum from infected mice is incapable of virus neutralization unless it is pretreated with ether or irradiated with ultraviolet light (228).

The immune complexes are filtered out of serum by the kidney and deposit on the epithelial side of the basement membrane of the glomeruli (230) in a manner identical to LCM virus immune complex deposition. Elution studies have shown that IgG from kidney contains anti-LDV antibody (230). The immune complexes have been demonstrated to fix complement (230,259); complement fixation elicits a mild proliferative glomerulonephritis. LDV antigens have also been detected

in association with the immune complexes (230, 259).

The mechanism of persistence of LDV is related to the interaction of the virus with a permissive cell type, an apparent abnormal immune response to the agent, as evidenced by production of non-neutralizing in addition to neutralising antibodies, and the circulation of infectious virus: antibody complexes (Figure 3). The virus is capable of replicating to relatively high titers in vivo without destroying infected cells. An abnormal immune response to LDV, resulting in production of non-neutralizing antibody may be related to the depression of T-lymphocytes which transiently occurs in animals following infection with the agent; there is no direct evidence to support this postulate, however. The hypothetical effect of T-cell depletion could be to decrease helper T-lymphocytes necessary for cooperation between B-lymphocytes and ultimate synthesis of neutralizing antibody. Cytotoxic T-lymphocytes may also be affected by LDV infection, rendering the animal incapable of clearing infected cells. The synthesis of non-neutralizing antibody could also have the effect of blocking the effectiveness of any neutralizing antibody produced as suggested by Notkins (226). Once the viremia is established, the virus can become complexed with IgG. These complexes eventually result in mild glomerulonephritis.

The macrophage has been postulated to play a major role in LDV persistence and in protecting the animal from severe

glomerulonephritis. By ingestion of immune complexes from serum, the amount of immune complex deposition in the kidney is reduced, thus lowering the potential for glomerulonephritis (201). Although LDV has been demonstrated by many investigators to be capable of replicating in a variety of phagocytic cells (primary mouse macrophages, mouse peritoneal macrophages, primary explants of reticuloendothelial cells) *in vitro*, no evidence exists demonstrating the ability of LDV to replicate in these phagocytic cells *in vivo*. By electron microscopy, LDV has been observed in peritoneal macrophages from infected mice, but it was unclear whether the virus was actually replicating in these cells or was simply being phagocytosed (225). Because the virus has been shown capable of replication in macrophages *in vitro*, it is likely LDV can replicate in these cells *in vivo*. Once macrophages have ingested the immune complexes, it has been postulated that the macrophage itself could become infected with the agent, thus providing an additional source for viral replication and fostering viral persistence.

Infection of the reticuloendothelial system could have far reaching effects on immune responsiveness to the agent as well as providing a reservoir for new virus production. It is well known that macrophages are an important cell type involved in stimulation of the immune response to T-lymphocyte dependent antigens (78,90,141,199,242,250,272, 273,298,332,333). Infection of the macrophage may alter the cell's ability to process and

present LDV-specific antigens to cooperating T-lymphocytes in a manner compatible with normal immune responsiveness. This may allow for an increased immuno-unresponsiveness on the part of the host (Fig. 3). LDV has been shown to affect the functional capacity of the immune system. Under certain circumstances LDV infection results in enhanced antibody production, depresses the induction of tolerance, depresses host- vs. -graft and graft- vs. -host cellular immunity, and depresses phagocytosis (227). Additionally, suggestive evidence has been obtained which indicates that LDV may induce immunosuppression in the host. It has been reported that certain tumors exhibit exhilarated growth when the host is also infected with LDV (331). Other investigators have found that certain protozoan infections of animals are potentiated in their severity by LDV infection (227). Notkins has suggested that these effects could be explained on the basis of LDV-induced immunosuppression (227). It is possible that these diverse effects arise from LDV infection of macrophages.

Eventual morbidity or mortality of the host is dependent on the level of circulating antibodies, viremia, quantity of serum immune complex, size of the immune complex, and the ability of the immune complexes to fix complement (203). In contrast to LCM infection, LDV infections are associated with high levels of circulating antibody in addition to virus-antibody complexes. In LCM virus

infection, the amounts of antiviral and non-neutralizing antibodies produced are quite low. The reason LCM virus infection results in such a low antibody response is because of the tolerance induced (see above) (Fig. 3). It is usually the case that LDV-infected animals with the highest levels of antibody develop the most severe immunopathologic disease. This probably correlates with the size of the immune complexes formed, rate of deposition in glomeruli, and ability of the complexes to fix complement.

Although LDV has been known to be an infectious virus of mice since 1960 (267), it has been only recently that LDV has been classified as a Togavirus (27). LDV differs in its physio-chemical properties from either the alpha- or flaviviruses of the Togaviridae. The agent has been known by several names, but each name refers to its ability to produce elevated levels of several plasma enzymes including lactic dehydrogenase, isocitric dehydrogenase, malic dehydrogenase, phosphohexose isomerase, glutamic oxaloacetic transaminase, and glutathione reductase (225,227). Elevated enzyme levels have been detected in the blood of a number of patients with malignancies and in several tumor-bearing mice; in fact, it was during experiments studying the relationship between enzyme elevation and tumor growth that LDV was discovered. The mechanism by which infection with LDV leads to elevated plasma enzyme levels appears to be due to an impaired clearance of endogenous enzymes from the

peripheral circulation and not due to leakage of enzymes from damaged cells or de novo enzyme synthesis (229). The mechanism of impaired clearance of enzymes seems to be related to LDV infection of the reticuloendothelial system (229). Clearly, infection of the reticuloendothelial system by LDV may result in many abnormalities in the host and possibly allow for maintenance of viral persistence.

LDV infection has been shown to not only result in immune complex disease but can also induce an age-dependent polioencephalomyelitis (ADPE) in C58 mice (193). APDE was initially discovered during studies of the immune response of C58 mice to the syngeneic leukemia I_b cell line. It was found that injection of inactivated I_b produced a paralytic central nervous system disease in immunosuppressed mice. The mice were immunosuppressed by normal aging or by immunosuppressive agents (75,204). It was determined that the I_b tumor line apparently harbored a particularly neurotropic strain of LDV (193). In retrospect, it is not surprising that the I_b leukemia line was contaminated with LDV in that 100 tumors and tumor-inducing virus stocks had clearly been shown to harbor LDV (227). The LDV-like ADPE agent has not been clearly shown to persist in serum as an immune complex but has been shown to replicate in mice of all ages. The mechanism of ADPE induction is currently unknown.

Aleutian Mink Disease:

Another naturally occurring persistent viral infection

resulting in chronic disease of its host is Aleutian Disease of Mink (AMD). The word "Aleutian" refers to a particular genetic variety of mink with the Aleutian coat color. Genetic factors appear to influence the severity of disease in that mink which are homozygous recessive for the Aleutian coat color genes (aa) develop particularly severe disease (203,257). AMD was first recognized to be caused by a virus (AMDV) in 1962 (142). Vertical or horizontal transmission of the infection has been demonstrated and once mink are infected, the virus persists for life (127).

AMD is a chronic, slowly progressive condition characterized by glomerulonephritis, arteritis, occasionally focal hepatitis, and most predominantly, a systemic proliferation of plasma cells resulting in a marked hypergammaglobulinemia (203,257). The hypergammaglobulinemia may progress into a monoclonal gammopathy which is secondary to the plasma cell proliferation (256). Death may occur from 2-24 months after infection; the genetic constitution of the mink playing a major role in delay of morbidity and mortality (113,203,237). The serum of infected mink is characterized not only by massive hypergammaglobulinemia, the IgG of which bears anti-AMDV specificity, but also by infectious virus-antibody complexes (203,256,257). Although anti-AMDV antibody is present in high levels in serum, the antibody in incapable of neutralization (259). Glomerular and arterial wall lesions have been demonstrated to contain

deposits of IgG and the C 3 component of complement; the IgG has been demonstrated to contain anti-ADV antibody in eluates of kidneys, and AMDV antigen has been demonstrated in acid eluates from arterial walls (114, 257, 258). AMD appears to bear certain pathogenic and immunologic similarities to LCM and LDV infections of mice; i.e., production of neutralizing and non-neutralizing antibodies, antigen-antibody complex formation, immune complex deposition in the kidney, and glomerulonephritis (Figure 3).

The hypergammaglobulinemia of AMD separates it from LCM and LDV infections. It has been shown that the hypergammaglobulinemia is directly due to overproduction of IgG rather than a defect in catabolism (256). The available evidence suggests that the hyperglobulinemia represents an extremely large humoral response to AMDV antigens (257), but one which is insufficient to neutralize the virus. Porter and his colleagues (257) have suggested that the macrophage plays a key role in AMDV persistence in AMD. Their mechanism is identical to the theoretical mechanism proposed above for LDV persistence (see Figure 3). In this model, the macrophage is thought to ingest circulating virus-antibody complexes, allow virus "reactivation;" replication within the macrophage results in

new virus production. The progeny virus is thought to complex with LDV-specific antibody and the complexes deposit in the glomeruli and upon the intima of blood vessels. Complex deposition triggers complement-fixation and results in vasculitis and glomerulonephritis.

Although an attractive model for the mechanism of AMDV persistence, no conclusive evidence has been obtained that AMDV is capable of replication in macrophages in vivo or in vitro. Immunofluorescence studies have demonstrated that AMDV antigens are present in the macrophage in vivo (257), however, this evidence alone is insufficient to prove that the virus is replicating in this cell type; positive fluorescence could well be due to phagocytosed antigen-antibody complexes. More recent studies by Hensen, et. al. (117) have demonstrated positive fluorescence mostly in cytoplasm of cells but also in nuclei. The fluorescence was distributed in intraperitoneally inoculated mink in lymph node and liver. The largest number of fluorescent cells in lymph nodes were present in the cortical areas. Fluorescent cells were rarely noted in the medullary areas. The cell type containing antigen in liver appeared to be Kupffer cells located perisinusoidally. Few fluorescent cells were observed in the portal areas (117). These investigators were not able to determine whether the fluorescing antigens in the cytoplasm of these cells represented phagocytosed antigen or antigen being produced within these cells.

Studies by Porter, et. al. (cf. Cho, ref. 45) have shown that AMDV can be propogated *in vitro* in cat cells. Predominant nuclear fluorescence was observed in these cells.

It is interesting that nuclear fluorescence was observed in AMDV infected cells *in vitro* and in macrophages taken from *in vivo* infections of mink. This suggests that AMDV has a nuclear-dependent phase during viral replication. Obviously, characterization of AMDV would be useful in determining the relevance of nuclear fluorescence.

Characterization of AMDV has been hampered by the relative difficulty of propagating AMDV *in vitro*. Several investigators have claimed that AMDV replicates in cultures of mink kidney and testis, mouse L, African Green monkey kidney, and WI-38 cell cultures (45). Other investigators have been either unable to confirm these findings or reproduce them. Most studies have used virus-antibody complexes isolated from serum as a source of virus for characterization. Conflicting reports have been published on the classification and nucleic acid type of the virus. Tenyan, et. al. and Yun, et. al. have suggested that the virus contains RNA as determined by the orcinol method and has an icosahedral structure with a diameter of 25 nm (cf. Cho, ref. 45). These results suggested that AMDV may be a member of the *Picornaviridae*. Cho, et. al. has suggested that ADV more closely resembles a parvovirus - a small, single-stranded DNA virus - by its physio-chemical properties (45).

In 1980, Bloom, et. al. were able to conclusively characterize AMDV as a parvovirus (20). These investigators showed that AMDV was 24-26 nm in diameter, had a density of 1.42-1.44 gm/cm^3 in CsCl, contained a DNA genome of 1.4 x 10^6 dalton molecular weight. By comparison of sedimentation profiles in neutral and alkaline sucrose gradients, the DNA appeared to contain only one of the two possible complementary strands (-DNA), and therefore concluded that AMDV most closely resembles the autonomous group of parvoviruses (like minute virus of mice). Therefore, the nuclear fluorescence observed by Hensen, et. al. and Porter, et. al. are consistent with a nuclear dependent phase during AMDV replication. Further experiments are needed to resolve the questions of whether AMDV can replicate in macrophages and the mechanism of the virus-nuclear dependent phase, in order to determine whether the hypothetical mechanism of AMDV persistence is valid.

The hyperglobulinemia and monoclonal gammopathy which result from ADV infection of Aleutian mink suggest that the virus-host interaction also results in a severe breakdown of normal immune regulation. It is conceivable that if AMDV is capable of replicating in macrophages that the normal antigen processing and presentation to other components of the immune system may be altered. Abnormal regulation of the immune response to AMDV may well result in abnormal clonal expansion of those plasma cells which secrete spec-

ific anti-AMDV antibody. Inappropriate immune regulation as a result of AMDV infection is further suggested by the observation of several characteristics of the disease, similar to autoimmune disorders of man, including the presence of Coombs positive hemolytic anemia, LE cells, anti-DNA antibody and antinuclear factor, in addition to the immune complex nephritis mentioned above (166).

Equine infectious anemia (EIA) in horses and visna-maedi in sheep are two additional infections in which persistence is a predominant feature. These two infections however differ from the others discussed thus far in many ways, and offer an additional mechanism of viral persistence. These two diseases are caused by C-type retroviruses (108,166).

Equine Infectious Anemia (EIA):

EIA virus (EIAV) produces a persistent viremia in horses (115). Infected horses develope an acute illness one to two weeks after primary exposure. Of those infected animals, some die early in the course of disease, and others recover from acute illness only to subsequently suffer from recurring episodic illness months or years later from which death can result. A few infected horses do not develop any clinical disease but become asymptomatic carriers of the virus. Active disease is characterized by severe hemolytic anemia, widespread lymphoproliferative lesions and hypergammaglobulinemia, hepatitis and glomerulonephritis (115).

The glomerulonephritis is due to deposition of virus: antibody complexes as evidenced by deposits of IgG with anti-EIAV activity and the C3 component of complement present in the glomeruli of infected horses (10). Viremia occurs in the presence of neutralizing and complement-fixing antibodies and some of this antibody is present in the form of circulating infectious virus:antibody complexes (150,212,213). The immunopathology of the glomerulonephritis observed in EIAV infected horses probably follows a mechanism analogous to LDV infection of mice and AMD.

The role of the macrophage in persistence of EIAV is probably more defined than in either LDV infection of mice or AMD. The early experiments of McGuire, et. al. (211) demonstrated that specific EIAV fluorescence could be observed in tissues following experimental inoculation of horses with EIAV. They observed viral antigens in a variety of tissues including the red, but rarely the white pulp of spleen, the medullary areas of lymph nodes, thymus, liver, kidney, bone marrow, lung, stomach, intestines, adrenal glands, pancreas, cerebrum, and cerebellum. Where they could determine in which cell fluorescence was observed, the macrophage was the most common. This was especially prominent in the liver, in which positive fluorescence was easily observed in Kupffer cells but not in adjacent hepatocytes. The time course of appearance of specific

fluorescence gave these investigators the impression that the macrophage was the first cell to exhibit positive fluorescence, and therefore the first cell type to support viral replication. In certain studies, the appearance of positive fluorescence occurred prior to the onset of viremia, indicating that presence of positive fluorescence in these cells may not have been due to phagocytosis of immune complexes. More direct evidence has been obtained in vitro, indicating that EIAV preferentially replicates in macrophages (56). Field isolates of wild-type EIAV will replicate only in horse macrophages and replicate only very poorly in other equine cell types. EIAV will not replicate in cells from species other than horses. In a natural infection, the virus would almost certainly initially replicate most easily in a permissive cell type (macrophage) and not in other non-permissive cell types. Thus, pathogenetic considerations, plus available experimental evidence strongly suggest that the macrophage is probably the primary site of replication in vivo, but infection and replication in other cell types may also occur.

The hemolytic anemia for which EIAV was named, is due to a complement-dependent lysis of erythrocytes (10,116, 209,210). During active hemolytic disease, horses have reduced levels of serum complement (C3). Binding of viral antibody to the surface of erythrocyte leads to complement fixation and lysis of the red blood cell (116,166,209). The

target on red blood cells to which anti-EIAV antibodies attach are presumably the hemagglutinin subunits of the virus (56). The depressed circulating complement (C3) levels are consistent with the presence of the infectious virus-antibody complexes, immune complex mediated glomerulonephritis, and the immune-mediated red blood cell destruction. Clearly, many of the clinical symptoms of infected horses and histopathologic appearance of lesions are consistent with immune-mediated mechanisms. Indeed, when Hensen and his co-workers treated horses infected with EIAV with cyclophosphamide, they did not develop severe hepatitis and glomerulonephritis (116). Hepatitis in EIAV infection is caused by cell-mediated lysis of infected hepatocytes (166), depression of cell-mediated immunity by cyclophosphamide treatment ablates immunopathologic injury to hepatocytes.

Perhaps the most unique feature of EIA is that many horses with the disease recover from the initial acute infection, but subsequently suffer from recurring episodes of clinical illness. Kono, et. al. (151) have found that these clinical episodes are associated with periods of rapid virus replication and are characterized by 3-7 days of fever and hemolytic anemia. The renewed viremia and antigen load prompts a vigorous immune response which sets in motion the same series of immunopathologic events that caused the clinical signs and lesions of acute EIA.

Interestingly, the studies by Kono, Kabayashi and Fukanaga (151) have suggested that each successive

linical episode is accompanied by virus differing slightly
n antigenicity from the original infecting agent and from
iruses isolated from previous episodes (56,151,203). This
rocess has been termed "antigenic-drift". Antigenic drift
ay provide a further mechanism for persistence of the virus
n vivo (Fig. 4). In this model infection of both permissive
nd non-permissive cell types by the retrovirus is envisioned.
n a permissive cell type, the RNA of the infecting virus
ill be reverse transcribed into a double stranded DNA
rovirus. The provirus is integrated into host cell DNA and
ubsequent progeny RNA molecules are transcribed from it.
ome of the RNA molecules will serve as mRNA, others as
enomic RNA. Virus maturation then occurs, and progeny
iruses bud from the cell surface. In the non-permissive
ell type, the proviral genome may be integrated, however,
he non-permissiveness of the cell type may produce either a
ranscriptional or translational block in the path of virus
aturation. Such intracellular restrictions on retroviral
eplication have been shown to exist, even though the
omplete viral genome may be present as integrated provirus
18,336,341). Intracellular restrictions may result in
ncomplete viral antigen synthesis, which, in turn, may
roduce a decrease of the immune response to the nonper-
issive, infected cell. Presence of the integrated genome
n a non-permissive cell provides for persistence of viral

genetic information within the host (Fig. 4). Following lytic growth of EIAV in the permissive cell (macrophage), the host mounts a normal immune response; a cell-mediated immune response is mounted to infected cells expressing viral antigen on their cell surfaces and a humoral immune response to soluble EIAV antigens and free virus. Immune complexes are formed as a result of the vigorous humoral response. Infection of the macrophages of the reticulo-endothelial system may ensue following phagocytosis of immune complexes from serum. A new round of viral replication may then occur in these cells resulting in the release of progeny virions bearing the same antigenic specificies as the parental virus with which they were infected. This theoretical mechanism allows for persistence of EIAV for a limited time, but only until the normal immune response has cleared the infection. Some infected horses die during this acute phase, presumably of immune-complex disease, however, some recover and subsequently develop episodic recrudescences characterized by a new round of viral replication. The integrated genome present in the non-permissive cell type may provide a source for new virus production. Perhaps through environmental factors or other mechanisms the latent genome present in non-permissive cells is capable of complete expression (Fig. 4).

A more attractive explanation of antigenic drift, however, is that the provirus undergoes mutation. If the

mutations occurred not only in those genes necessary for complete viral expression but also in those genes coding for the major envelope glycoproteins of the virus, the new virus produced would be, to at least some degree, antigenically different from the parental virus with which the cell was initially infected. A new round of infection could then occur resulting in reinfection of new permissive cells as well as non-permissive cells culminating in a new episode of disease. Circumstantial evidence has been obtained which indicates that persistent infection of a non-permissive cell type can occur in EIA. Crawford, et. al. (56) were able to develop an in vitro model for the persistent infection in vivo. These investigators established persistent infection of equine fibroblasts and characterized the production of EIAV and EIAV antigens during the course of infection. These studies suggested that it is at least possible for a non-permissive infection to exist but did not directly address the question of whether lysogenous infection of non-permissive cell type was directly involved with the pathogenesis of disease. Further analysis of the virus-host interaction in EIA infection in vivo and in vitro will be necessary to completely sort out the mechanisms of persistence, and the generation of the variants.

It is unclear what role the antigenic variants of EIAV may play in the episodic nature of chronic EIA.

The studies by Kono, et. al (151) have shown that a new antigenic variant becomes predominant in the blood in concert with each new clinical cycle. This permits a temporary escape of the new variant from the immune system, "primed" by the previous variant. The appearance of antibody to the new variant in a few days, apparently re-establishes immunologic control, and the viremia subsides. Available evidence indicates that little antigenic relatedness exists between the viral isolates (56,151), and therefore cross-protection by antibody is insufficient to prevent reinfection during each new clinical cycle. What mechanism (s) is(are) responsible for the eventual cessation of clinical cycle seen in most horses is(are) unknown, but probably relates to the cellular immune response to infected cells rather than the humoral response.

Visna Virus Infections:

Another persistent viral disease of animals in which antigenic drift has been demonstrated to occur is Visna-Maedi of sheep (214,215). In 1954, Bjorn Sigurdsson introduced the term "slow virus infections" to describe the unusual characteristics of several diseases that appeared in Iceland during 1930-1950 (299). These diseases included Visna and Maedi and Scrapie (Rida). Scrapie is a degenerative non-inflammatory disease of the central nervous system caused by an unconventional agent and has been described above. Visna ("wasting") and Maedi ("shortness of

breath") refer to the neurological and pulmonary manifestations, respectively of infection of sheep caused by a conventional agent. Visna-Maedi, although first observed in Iceland, has been described elsewhere in the world and is known as Zoegerziekte in the Netherlands and as Progressive Pneumonia in the United States.

The viruses that cause Visna/Maedi/Zoegerziekte/Progressive Pneumonia belong to the Lentiviridae subfamily of the Retroviridae. The other subfamilies of the Retroviridae include the RNA tumor viruses (Oncoviridae) and the foamy viruses (Spumaviridae) (158). The lentiviruses are non-transforming and are not associated with neoplasm but rather with slowly progressive inflammatory disease. The lentiviruses are serologically distinct for both type and group specific antigens from the other retroviruses and EIAV (311) and further, do not share any nucleotide sequence homology with other members of the oncornavirus subfamilies (109, 306). The lentiviruses have a similar but not identical mechanism of replication to the other members of the Retroviridae. Lentiviruses use a proviral DNA intermediate during replication; however, replication does not require cellular DNA synthesis or mitosis. In contrast to most other retroviruses, there appear to be no endogenous lentivirus genes in uninfected cells (104).

In nature, Visna virus appears to be transmitted horizontally from animal to animal; lambs receive virus from

ewes via the milk, older animals transmit the virus by respiratory aerosols or infected saliva. Since endogenous lentiviruses have not been detected (104) and transplacental transmission has not been demonstrated experimentally (68), it is unlikely that vertical transmission occurs.

Experimental infections of sheep have elucidated the pathogenesis of visna (cf. Haase, et. al., ref. 106). Following intracranial inoculation, the virus replicates in the choroid plexus and ependymal cells of the central nervous system and elicits a host-immune response. Inflammatory cells begin to infiltrate the infected area a few days after inoculation, and the cellular response peaks at about 2 weeks, decreasing to lower levels in about one month (103). The humoral immune response is characterized by the presence of complement fixing antibodies appearing a few weeks after inoculation, which rise to maximum titers in a few months, and stay at high levels throughout the course of the disease. Neutralizing antibodies appear later than complement fixing antibody (between 1-4 months post-inoculation) and also remain at high levels throughout the course of infection (247). There are also non-immunoglobulin components in serum and cerebrospinal fluid capable of neutralization (328).

The incubation period of experimentally infected sheep is irregular, but slow, ranging from 2 months to over 10 years; the clinical phase follows a protracted course gen-

erally extending over several years. The onset of the disease is insidious. Slight aberation of gait, especially of the hind quarters, is the first sign. Unnatural tilting of the head and, in rare cases, blindness, may be observed. The disease progresses to hemiparesis or even total paralysis. Unattended animals will die of malnutrition. If animals are helped to obtain food and water, they may survive for longer periods of time. Following inoculation, a viremia occurs which disseminates virus to distant organ systems including the reticuloendothelial system and the lungs. The inflammatory response to infection of the lungs results in progressive pneumonia. Animals become dyspneic and with time, respiration becomes more difficult. Animals with Maedi often die from acute bacterial pneumonia. The primary cause of clinical signs in Visna-Maedi is immunopathologic in nature.

Visna virus replicates poorly in experimentally inoculated animals. Narayan, et. al. examined recovery of visna virus from a variety of tissues following intracerebral inoculation of fetal lambs. These investigators found that virus recovery from choroid plexus, brain, lung, spleen, kidney and the buffy coat was much more efficient from tissue explants than from tissue homogenates (217). Virus recovery was possible for weeks after infection, although the frequency of successful virus recovery from explant cultures decreased after the first two months post-infec-

tion. When similar studies were performed using adult sheep, virus could be recovered from tissue explants for one year after intracerebral inoculation, however, no cell free virus was found between 10 days and one year post-inoculation (217). In contrast, visna virus causes lytic, productive infection in sheep cells in vitro (104). Thus, it would appear that Visna virus replication in vivo is restricted, compared to virus replication in vitro. Furthermore, despite the vigorous inflammatory and humoral immune responses mounted by the host, visna virus is capable of persisting in vivo throughout the course of infection.

The fact that Narayan, et. al. (217) could efficiently recover virus from tissue explants but not from tissue homogenates suggested that the virus genome was present in tissue but did not become fully expressed or activated until cells were cultured in vitro. Haase, et. al. (105) examined the state of visna virus in tissue from experimentally inoculated sheep. These investigators found that by electron microscopy, no virions could be observed in choroid plexus tissue and that tissue homogenates contained no infectivity. However, explant cultures of choroid plexus tissue yielded virus. Immunofluorescence of choroid plexus tissue was negative. Using in situ hybridization to detect visna virus DNA in infected cells, these investigators found a few cells containing viral genome equivalents. These data suggested a lysogenic state of infection, whereby Visna

virus DNA was introduced into and stably maintained in at least a few cells. Such latently infected cells may go undetected by the immune surveillance system of the host and would serve as a reservoir for viral genetic information. However, because animals with Visna do develop neutralizing antibodies during the course of disease, some virus replication (lytic production of virus) must also occur. Perhaps these occasional cells do not restrict lytic growth or the virus is capable of overcoming the restriction.

Narayan, et. al. (217) demonstrated that peripheral blood leukocytes of infected sheep contained visna virus DNA. When peripheral blood leukocytes were inoculated with visna virus no productive replication occurred, however, virus could be recovered from these cells by co-cultivation techniques (217). It is possible, therefore, that peripheral blood leukocytes may harbor proviral visna DNA and serve as an "effector" for dissemination of virus throughout the animal. For virus to infect other tissues from a latently infected peripheral blood leukocyte requires that the virus-cell restriction of viral gene expression be lifted, so that the peripheral blood leukocyte can release progeny virus into its immediate environment. No direct evidence has been obtained to date indicating that the peripheral blood leukocyte plays this role in the pathogenesis of Visna, however.

The nature of visna virus persistence <u>in vivo</u> would appear to involve a virus-host cell interaction in which the

provirus is maintained within a certain number of cells that can evade the immune system. Haase et. al. (105,106) have found that many cells of the choroid plexus of an animal infected with 10^7 pfu virus contained viral DNA, as detected by *in situ* hybridization, at about the same proportion as scored by infectious foci experiments (105). Only a small fraction of cells containing viral DNA were found to contain viral-core specific antigen (P30) and major virion glycoprotein antigen (gP 135) (106). Haase, et. al. (106) have developed a quantitative method for determining the amount of visna viral RNA and DNA per cell. During the course of productive lytic infection *in vitro*, viral RNA increased from a few copies per cell to several thousand copies per cell by the end of the growth cycle (106). These investigators have demonstrated that only a few cells in sheep choroid plexus contained viral RNA (but many cells contain viral DNA). These data suggest that viral DNA is maintained in cells *in vivo* but expression of viral genetic information is blocked at the transcriptional level (Fig. 4). Those cells demonstrated to contain viral RNA may be the source of the small amount of free virus found in infected tissues (106). The few cells present in infected animals expressing viral RNA could be thought of as "productively infected" and would be the targets for immune lysis. The vast majority of cells, however, maintaining the viral genome in a lysogenous state, would be immunologically

"silent" and would therefore not be attacked by the immune system.

Antigenic drift has also been shown to occur in Visna (215,216,217). Following intracerebral inoculation of a sheep with one strain of visna virus, three isolates of virus were obtained from peripheral blood leukocytes during the course of infection. The three isolates obtained were compared with the parental virus by mapping of T_1 resistant oligonucleotides (46). These investigators found that the three isolates were antigenically distinct from the parental virus and from each other (216). The antigenic variants were found to contain mutations in the glycoprotein gene that codes for the antigen to which the neutralizing was directed (46,216,294). Interestingly, although antibodies to the parental virus and the three antigenic variants of visna virus appeared to develop in sequential order (217), the parental and variant strains could be isolated contemporaneously from peripheral blood leukocytes (216).

A theoretical mechanism for Visna virus persistence is outlined in Figure 4. Growth of the virus in a non-permissive cell type results in maintenance of viral genome equivalents. Complete viral expression is limited by a transcriptional block which results in incomplete mRNA formation and consequential incomplete synthesis of viral antigens. This results in a decrease or an absence of an immune response to such lysogenously infected cells; only those cells per-

missively infected are attacked by the immune system. Mutation of the viral genome in the 3'-end of the genome may result in alteration of the major glycoprotein of the virus. Virus released from such cells will not be neutralized by antibody directed to the parental virus. These antigenic variants may then infect other permissive and nonpermissive cell types.

The role of the antigenic variants in persistence of Visna virus, dissemination of virus to distant organs, and evolution of chronic progressive disease is unclear for two reasons. First, all Visna virus variants were derived from peripheral blood leukocytes explanted in culture, indicating that the variant strains are not found extracellularly. Secondly, unlike the antigenic variants of EIAV, Visna virus variants do not arise sequentially replacing one another throughout the course of infection. Therefore, although antigenic drift has been demonstrated to occur during Visna virus infection in vivo, it is unclear what role the variant forms of the virus play in the maintenance of persistence as it is clear that the parental virus is capable of persisting in the host despite the generation of viral variants and a vigorous immune response. It is perhaps conceivable that the variants may play a role in spread of Visna virus throughout a population of sheep in a manner reminiscent of influenza epidemics in populations of humans or borrelia or malaria infections in individual animals. Clearly, further

investigations are necessary to determine what role, if any, antigenic variants of visna virus play in the pathogenesis of disease, maintenance and establishment of persistent infections in a single animal, and what the relationships are between the host and the lysogenously and lytically infected cells.

Herpes Virus Infections:

In addition to the lentiviruses, the herpesviruses have been demonstrated capable of establishing a latent persistent infection *in vivo*. Of all members of the genus *Herpesvirus*, Epstein-Barr (EBV) and herpes simplex viruses types 1 and 2 (HSV-1, HSV-2) have been the most studied with respect to their ability to induce latency, persist *in vivo* and *in vitro*, and to establish the relationship between latency and the appearance of disease. HSV is responsible for recurrent infections in man, most often taking the form of periodic cutaneous, oral or genital mucosal infections, but the virus has also been shown to be responsible for serious generalized infections in newborns or to produce meningitis, myelitis or encephalitis in adults (12). EBV is associated with infectious mononucleosis, (110), African Burkitt's lymphoma (111) and nasal pharyngeal carcinoma (110).

Although HSV is capable of lytic replication in a wide variety of cell types *in vivo* and *in vitro*, the sensory and autonomic ganglia of animals and man are the sites where HSV

has been demonstrated to persist in a latent form (15,17,272, 263,308) EBV preferentially infects B-lymphocytes in vivo (197), and in vitro (198). What is known about the pathogenesis of HSV infections, establishment and maintenance of persistence and latency will be outlined below.

Much of the information on the pathogenesis of HSV infections has been obtained from animal models. In experimental animals following inoculation by various routes, the virus centripetally spreads along neural pathways (14,51,97,98,99,138) and infects the appropriate dorsal or autonomic ganglion (262,263). The acute stage of ganglionic infection is defined by the presence of infectious virus in cell-free ganglion homogenates and relatively easy visualization of viral particles by electron microscopy within the ganglion (12). After the productive infection disappears the chronic or latent stage is established; during this stage the virus can be demonstrated only by explantation of ganglia or co-cultivation with suitable indicator cells (11, 12,15,17,149,309,310) Walz, et. al. (337) have shown by infectious center assays that the fraction of ganglion cells harboring the virus decreases from 1% during the acute stage to 0.1% during the chronic stage. In this regard it is perhaps interesting that Baringer and Swoveland (16) demonstrated a single abnormal ganglion cell in the entire trigeminal ganglia removed from a rabbit latently infected with HSV. These very rare ganglion cells were surrounded by

a thick cuff of mononuclear cells. The ganglion cells contained extensively vacuolated cytoplasm containing a few enveloped HSV particles; nuclei contained HSV capsids with cores of variable density (12).

The stereotypic recurrent mucocutaneous lesions produced by HSV are probably due to reactivation of the latent ganglionic infection. A variety of environmental factors have been demonstrated to provoke recrudescences (Fig. 4), including exposure to sunlight, allergic reaction, trauma, menstruation and fever; hence, the two common terms for mucocutaneous herpetic lesions, "fever-blisters" and "cold sores". Iatrogenic manipulation of the centrally projecting sensory fibers of the trigeminal ganglia has also been demonstrated to provoke HSV lesions (40,41,77) (Fig. 4). Reactivation of latent ganglionic HSV infection can also be achieved in vitro. Openshaw, et. al. (232) demonstrated that explantation of latently infected ganglia in vitro resulted in the formation of progeny after 48 hrs of culture. Reactivation occured in this system in the presence of neutralizing antibody. Reactivation has been demonstrated to occur in vivo in the presence of antibody following irradiation or cyclophosphamide treatment of latently infected immunocompetent mice (232). Earlier attempts by Stevens and Cook (309) to reactivate latent HSV infection in vivo were unsuccessful; the recent studies by Openshaw, et. al. indicate that the host immune response plays a critical role

in controlling the acute phase of the ganglionic infection and further suggest that depression of the host's cellular immune response is an important factor for reactivaton of the virus in the presence of neutralizing antibody.

Although little is known of the virus-host relationship during the acute phase leading to the establishment of latency, even less is known about the state of the latent viral genome in infected ganglion cells. The central unsettled question in this system is the state and activity of the viral genome during the chronic state. Two possibilities have been put forth to describe the state of the viral genome, "static" and "dynamic" viral latency (71, 148,270,307). Static viral latency is a condition in which the genome is maintained in a non-replicating state. Dynamic latency, on the other hand, is a state in which the virus is envisioned to multiply at a slow rate, producing a persistent infection in which infectious virus cannot be detected by standard techniques. The latter is reminiscent of Visna virus infection of sheep choroid plexus as described by Haase et. al., (106), outlined above.

Recently, several groups of investigators have examined acute and latent HSV infection <u>in vivo</u> to determine the level of viral DNA and viral specific-mRNA present in cells. Puga, et. al. (264) have found viral DNA present in acutely infected cells at a level of 1.2 to 2 genome equivalents per cell. Viral specific mRNA was found at a level of 0.1-0.2

equivalents per cell during the acute infection but at less than 1 equivalent per 2000 cells in latently infected ganglia. These studies were performed by following re-association kinetics of ganglionic DNA and RNA mixed with a probe consisting of ^{125}I-labeled viral DNA. These studies suggested that the acute phase of ganglionic infection resulted in normal transcription of viral DNA and RNA and production of progeny virions. This was confirmed by isolation of infectious virus from homogenates of acutely infected ganglia. During the latent infection, however, there was an apparent decrease in the number of cells harboring latent viral DNA and an almost total absence of viral mRNA transcription. Galloway et. al. (93) used a nick translated ^{3}H-DNA probe specific for HSV-2 RNA to examine the amount of viral specific RNA present in the paravertebral ganglia removed from humans at autopsy. The ganglia were frozen upon removal, sectioned and hybridized to the ^{3}H-HSV-2 DNA probe <u>in situ</u>. These investigators found that 0.4-8% of the neurons present in the ganglia were expressing HSV RNA; the RNA detected was in association with the Nissl bodies indicating that the neurons were elaborating HSV-specific RNA. Other ^{3}H-DNA probes from other viruses, prepared in the same manner, did not hybridize to any cells of the ganglia. Taken together, these results indicate either that many latently infected ganglion cells contain HSV viral DNA and only some cells express viral genes, or that only some neurons are latently infected. In

either case, it would appear that the latent state involves an host-virus interaction which is non-permissive for viral mRNA transcription (Fig. 4). By analogy to Visna virus infection of sheep choroid plexus, one would predict that if the hypothetical transcriptional block is present, that few or no viral antigens should be expressed in latently infected ganglia.

Levine, et. al. (163) established a persistent HSV-1 injection *in vitro* using B 103 neuroma cell lines. Following a brief lytic infection, surviving cells ceased to produce progeny and appeared identical to uninfected neuroma cell lines; both infected and uninfected lines had the same growth rate. Interestingly, the persistently infected neuroma cells did express certain, but not all HSV-1 antigens. Those antigens which were missing in the persistently infected cell line *viz. a viz*. productively infected cell line, were those antigens shown to be responsible for complement mediated-immune cytolysis. Levine, et. al. demonstrated that persistently infected neuroma cell lines were totally resistant to complement mediated-immune cytolysis *in vitro* (163). These results suggested that the latent infection may be one in which only some latently infected cells express certain viral genes. Limited expression of viral antigen has been demonstrated in established latent ganglionic infection. Using well characterized antisera to early and immediate-early HSV antigen,

Rajcani, et. al. were unable to demonstrate positive immunofluorescence of immediate-early and early antigens in serial sections of ganglia from rabbits with established latent infection (Rajcani, Matis, Field, unpublished observations). After latently infected ganglia were explanted in culture, however, positive fluorescence of early and immediate-early HSV proteins was detected; appearance of immediate-early and early antigens correlated with "explant-reactivation" (Rajcani, Matis, Field, unpublished observation).

Although available evidence suggests that maintenance of persistent latent HSV infections in vivo is associated with a transcriptional (and subsequent translational) block (see Figure 4), little information is available which sheds light on the establishment of latency. Recent evidence has been obtained which implicates both viral genetic determinants and the host's immune response as factors which may be involved in establishment of latency.

Tenser, et. al. (318,319) have determined that HSV thymidine kinase (TK) expression may be required for establishment of latent ganglionic infection. These investigators infected rabbits with TK+ and TK- strains of HSV by the corneal scarification technique (12). HSV TK+ strains were capable of replication in the cornea and the trigeminal ganglia, whereas HSV TK- strains were unable to replicate in the ganglia, but could replicate in the cornea. These data

indicate that the expression of TK is apparently required for acute replication in the ganglia, a necessary prerequisite to establishment of latency. Fong and Scriba (88) used ^{125}I-deoxycytidine as a substrate for measurement of TK activity in latently and acutely infected ganglia of guinea pigs. They could not detect viral TK expression during latent infection. It is possible that TK expression is required for establishment of latency, but once established, the TK gene need no longer function.

The host's immune response during acute ganglionic infection has been implicated by Sokawa, Ando and Ishihara (301) to play a role in the conversion from the acute phase to the latent phase of infection. These investigators examined ganglia during the acute and latent stages of infection for the presence of 2', 5'-oligoadendylate synthetase and interferon activities. The anti-viral substance, interferon, has been demonstrated to induce cells to produce at least 2 enzymes: one is 2', 5'-oligoadenylate synthetase, which catalyses the formation of 2', 5'-oligoadenylate from adenosine tryphosphate (ATP) in the presence of double-stranded RNA; the other enzyme is a double-stranded RNA-dependent protein kinase, which can inactivate the eukaryotic protein synthesis initiation factor, eIf-2, by phosphorylation (7). Their data indicated that the acute phase of HSV replication in infected ganglia was suppressed by interferon production; acute-phase suppression occurred

before antibody synthesis had begun (301). Although unconfirmed, this report indicated that the host's immune response may play a role in establishment of latency. It is unclear how the immune system (interferon production, antibody synthesis, and cellular immunity) may interact with an infected ganglion cell to result in an alteration of viral gene expression, and the conversion from an acute to a latent state of infection.

Defective Interfering Particles, Viral Persistence and Chronic Disease:

Although there are numerous other persistent virus infections of man and animals, the examples given above and diagrammed in Figures 2, 3 and 4, serve to illustrate the variety of extant and hypothetical mechanisms by which viruses may persist in a single animal. One mechanism of persistence which has not been described here, but is diagrammed in Figure 4, is the generation of defective interfering particles contributing to the establishment and maintenance of persistence. Defective interfering (DI) particles are defined as viruses which are defective, in that they cannot replicate in the absence of a wild-type helper virus, and interfere with the replication of wild-type virus in cells co-infected with DI particles (125). DI particles may be involved in viral persistence by interacting with the host cell and wild-type virus in such a way as to prevent normal viral-lysis of the infected cell, and

thus allow cell survival. Viral genetic information, albeit incomplete due to the defectiveness of the DI particle, could be preserved and thus persist.

Huang and Baltimore first postulated that DI particles might play a role in viral persistence (124). Although DI particles have been used to establish persistent infections in vivo (144) to date, however, evolution of DI particles in vivo during an infection has not been convincingly demonstrated. Therefore, their role in establishment and maintenance of a natural persistent infection in vivo remains unestablished.

DI particles have been generated in vitro by high multiplicity passage of viruses of a number of classes (cf. Huang and Baltimore, ref. 125), and have been shown to play some role in establishment and maintenance of persistence in vitro (2,122,123,145,255,274,290,296,310). The precise mechanism of DI particle regulation in establishment and maintenance of persistent viral infections in vitro have not been clearly defined and differences exist among the various virus-cell interactions studied. Other viral particles which may be involved with persistence in vitro and in vivo are temperature-sensitive mutants (261).

Role of Viral Persistence in Nature:

In view of the fact that viruses of a wide number of classes cause periodic epi- or pandemics, it is germane to consider what advantage(s) persistence in a single animal

may provide to the virus with regard to the population as a whole. A virus which persists in a single animal, without production of progeny, is an evolutionary dead-end for the virus; death of the host will lead to cessation of viral spread throughout the population. Thus, persistence within an individual can only "serve" the virus if the persistent viral infection allows for vertical or horizontal spread of the virus to another susceptible host.

Viruses capable of integration may pass viral genetic information from generation to generation by infection of the germ cell line; indeed, comparison of inter- and intra-species inherited oncoraviral gene sequences has led to a form of biochemical evolutionary taxonomy (cf. Vogt, ref. 336). Those viruses incapable of integration, do not share the advantage of vertical transmission with the retroviridae. For nonintegrating viruses, the ability to persist in an infected individual can offer the singular advantage that, if progeny are formed during the persistent state, the host is a walking carrier of the virus, provided the virus is released or excreted from the host. Thus, the persistently infected individual may offer an advantage to the virus by offering a reservoir of viral genetic information between episodes of large scale infections of the entire population. Antigenic drift or shift of a virus may provide a further mechanism for persistence of a virus within a population by allowing a temporary "escape" from herd imm-

unity. Although the list of viruses capable of producing persistent infection in an individual animal is increasing, it is generally not known what the frequency of induction of persistent infection is during a naturally occurring epidemic. In the laboratory, an investigator can manipulate an experimental animal model to yield a high proportion of persistently infected animals through unnatural routes of administration of excessively large quantities of virus (intracranial inoculation), by the use of immunologically compromised hosts, or by the use of an animal which is not the natural host for the virus. Furthermore, an investigator may choose to examine the nature of a persistent viral infection in vitro using nonpermissive cell lines, unusual temperatures for incubation, the use of a variety of metabolic inhibitors, etc. But given these limitations, the study of persistent viral infections is warranted because of the far reaching effects a persistently infected host could have on a naive population.

Possible Mechanisms of Theiler's Virus Persistence and Chronic Disease Production:

Although Theiler's viruses have been know to persist in the central nervous system of mice following experimental inoculation since the original studies by Theiler in 1937, the mechanism of viral persistence remains unknown. Moreover, the relationship between viral persistence and central nervous system demyelination, both features of chronic

Theiler's virus disease, is a matter of conjecture at the present time due to the paucity of data concerning the interrelationships of these phenomena. Any hypothetical model for Theiler's virus persistence, must address each of the empirical observations which have been made since 1937 regarding persistence of the virus in vivo.

The salient features of Theiler's virus persistence include: a low level of continuous viral replication within the central nervous system during the chronic stage of disease, continued free virus production in the presence of relatively high serum neutralizing antibody titers, low or no growth of the virus in extraneural tissues, the apparent absence of a viremia during the acute and chronic stages of disease, the potentiation of neuronolytic infection during the chronic phase of disease by immunosuppressive treatments, the apparent absence of viral antigens during the chronic demyelinating phase of disease, the apparent absence of any degenerative changes in oligodendroglial cells during the chronic phase, the apparent rarity of infected cells as demonstrated by the failure to demonstrate viral inclusions in any brain cell during the chronic phase, the fact that complete attenuation of the encephalitogenic potential of strains of Theiler's virus which can cause persistent infections in vivo is completely attenuated when the same strains are adapted to tissue culture, and the correlation that virus recovered from persistently infected animals

retains its encephalitogenic potential to induce acute disease as measured by subinoculation of central nervous system tissue homogenates from persistently infected mice into other suckling or weanling mice. Furthermore, it would be useful in constructing a hypothetical model for Theiler's virus persistence to interrelate persistence and demyelination. The data of Lipton, et. al. (60,167,171,173,174) suggested the possibility that chronic demyelination was immune-mediated, and that the ability of a strain of Theiler's virus to persist in vivo is related to its ability to produce only small plaques when adapted to tissue culture (168,170). These investigators have not reported any relationship between viral persistence and apparent immune-mediated disease.

Other factors which must be considered in developing a hypothetical mechanism interrelating viral persistence and demyelination is the evidence that certain strains of Theiler's virus (eg. WW strain) are capable of replication in neurons, oligodendroglial cells and astrocytes in vitro (339) and in vivo in suckling mice (245).

It is clear from these observations that most strains of Theiler's virus demonstrated to persist in vivo are immunogenic, elicit an apparently normal immune response, do not persist in vivo as circulating antigen: antibody complexes analogous to LDV and LCM infections of mice, EIAV infections of horses, AMDV infection of mink and rubella

virus infections in humans resulting in PRP. Reverse transcription of Theiler's virus RNA into a DNA proviral intermediate and integration are implausible mechanisms; antigenic drift of the virus during the course of infection has not been reported.

Because picornaviruses are notoriously lytic, and because central nervous system Theiler's virus persistence is associated with continued free virus infection, a hypothetical model for persistence probably involves a relatively permissive cell type. These putative permissively infected cells are probably few in number accounting for the paucity of positive immunofluorescence and the failure to demonstrate viral inclusions in brain cells during the chronic phase of disease. During the course of the acute phase of Theiler's virus infection, free virus titers begin to decline approximately coincident with the rise in serum neutralizing antibody titers. Furthermore, it is during this period that mononuclear cells begin to appear in large numbers in the white matter. Presence of these cells heralds the beginning of the chronic demyelinating phase of the disease.

The inflammatory nature of demyelination produces a decrease in the "tightness" of the vascular-endothelial blood-brain barrier. Serum antibodies would be capable of entering the central nervous system through vascular walls of increased permeability. Influx of IgG would account for

the decrease in free virus titers recoverable during the chronic phase of disease. The resulting persistent infection could be thought of as analogous to a persistently infected carrier culture in vitro where persistence is maintained by controlling an acute, lytic infection of the entire monolayer of cells, by maintaining the culture in the presence of specific immunoglobulin (82). Removal of immunoglobulin allows for infection of more cells in the culture.

The potentiation of neuronolytic infection in vivo following immunosuppression treatment of persistently infected mice supports this concept of Theiler's virus persistence as a "static-state" of infection, whereby excessive neural spread within the central nervous system is controlled or eliminated by serum IgG. One prediction of this model would be that subinoculation of a susceptible naive mouse with central nervous system tissue homogenates of a persistently infected mouse, would result in acute viral replication concomitant with acute disease. This is in fact what has been demonstrated to occur; homogenates from chronically infected mice do not appear to contain a virus which has been altered in any way so as to produce only chronic disease upon in vivo subpassage.

The slow progressive nature of chronic Theiler's virus disease could be due to the relatively slow cytolytic cell-to-cell spread of those progeny virions not neutralized by

antibody. The slowly progressive, static-infection would also elicit a cellular immune response to those few infected cells. In this sense, demyelination would be immune-mediated; the target cells for cytotoxic T-lymphocytes and macrophages would be virus infected cells. Primary demyelination, resulting from direct lysis of an oligodendrocyte, and secondary or immune-mediated demyelination would occur simultaneously. Abrogation of humoral and cellular immunity by severe immunosuppressive treatment would provide for unrestricted spread within the central nervous system and subsequent renewed neuronolytic infection, reminiscent of the acute phase of disease in a naive host.

Although the static-infection model of Theiler's virus persistence explains many of the characteristics of persistent infection in vivo, it does not offer an explanation for the observation that during the course of infection, lesion distribution changes from gray matter during the acute phase to white matter during the chronic phase. This shift, defined histologically as polioencephalomyelitis during the acute phase and demyelinating myelitis during the chronic phase correlates with the signs observed in infected animals throughout the course of infection, and is pathognomonic for Theiler's virus infection. If one assumes that all brain cells are equally permissive for replication of Theiler's virus, the static-infection model would predict that during the chronic phase of disease, when antibody levels are high, slowly progressive involvement of infected

cells would occur and one would see progression of gray and white matter diseases simultaneously. But in fact, what one observes, is an apparent restriction of lesions to white matter only; particularly white matter of the spinal cord. If persistence and demyelination are interrelated, a hypothetical model for Theiler's virus persistence must explain the shift of lesion distribution.

One mechanism which could explain this shift involves the concept that not all brain cells are equally permissive. In this model, the neuron could be thought of as the most permissive cell type whereas the oligodendrocyte and other brain cells as less permissive; all cells allow for viral replication but those cells which are the most permissive allow for the most efficient production of progeny. Following infection, the virus would be able to replicate in permissive (neurons) and nonpermissive (oligodendrocytes) cells. Complete and rapid viral replication would occur in permissive cells, resulting in high levels of free virus production and expression of viral antigens. These cells would undergo rapid lysis, result in the release of relatively large quantities of the chemical mediators of inflammation triggering the local inflammatory response. The microglial response and chromatolytic appearance of neurons during this phase would give rise to characteristic lesions of polioencephalomyelitis. Replication in less permissive cell types would give rise to lower levels of free virus

production, less expression of viral antigen, and perhaps allow for a longer survival of the cell. Rapid clearance of the highly permissive cells through neuronophagia by microglial cells, and neutralization of free virus by the rising level of antibodies which mark the end of the acute infection, would tend to decrease the number of highly permissive infected cells, thus preventing the spread of free virus to other uninfected, highly permissive cells. The less permissively infected cell types would be the only remaining cells capable of replicating the virus; the presence of IgG in the extracellular milieu would further prevent spread of free virus to other cells.

The cellular immune response to the virus would then be "shifted" to those less permissive cells infected by the virus. If the neuron represented the highly permissively cell type and the oligodendroglial cell, the less permissive cell type, a shift of the site of viral replication would also correlate with a shift of the localization of the inflammatory response, thus accounting for the change in lesion distribution throughout the course of infection. Continued replication of the virus in oligodendroglial cells in the presence of IgG, would prevent widespread dissemination of virus. Only through a cell-to-cell spread would progeny virus be capable of establishing sites of new infection. Eventual lysis of the oligodendroglial cell would give rise to primary demyelination; the cellular immune

response to these infected cells could give rise to secondary immune-mediated demyelination.

This model, termed the "relative-permissive, static-state" model of Theiler's virus persistence accounts not only for low levels of persistent cell-free virus present during the chronic phase of disease, and the shift of lesion distribution from gray matter to white matter, it also offers a partial explanation for the paucity of viral antigen detected late in infection and the failure to detect viral crystalline arrays in any cell type during chronic disease. Acutely infected cells that are highly permissive for the virus would contain large crystalline arrays whereas less permissive cells infected with the agent would probably not. Furthermore, this model predicts that the effect of immunosuppression would be to allow sufficient spread of progeny to highly permissive cell types, by decreasing the amount of neutralizing antibody in the extracellular milieu. Renewed neuronolytic infection would ensue, resulting in a return to grey matter disease. The data of Lipton, et. al. (173,174) support this concept.

The encephalitogenic potential of certain strains of Theiler's viruses (TO or TO-like) is lost following tissue culture adaptation; intracerebral inoculation of these strains results not in a biphasic disease, but rather produces only the chronic form of disease accompanied by viral persistence. This observation allows further investigations

of hypothetical mechanisms of Theiler's virus persistence *in vivo*. Two possible explanations for the attenuation of the encephalitogenic potential of these strains can be imagined: selection of a particular variant during the adaptation process, and/or mutation of the virus during tissue culture passage. The selection hypothesis is one in which two morphologically and probably antigenically and biochemically very similar variants exist in the brains of acutely infected animals. During tissue culture adaptation, a process requiring blind-subpassage until cytopathic effect is produced, that variant originally capable of producing encephalomyelitis *in vivo*, is lost, leaving only that variant capable of producing demyelinating myelitis and establishing persistence. The mutation hypothesis is one in which a mutation occurs in the virus(es) during the adaptation process. The resulting progeny could arise by two different pathways. The first considers the virus present in an acutely infected mouse brain to be homogeneous and that the putative mutation provides the virus with an ability to produce cytopathic effect in culture and coincidentally to produce only demyelinating myelitis *in vivo*. A second pathway involves the presence of multiple variants of the virus to exist in mouse brain, and that a mutation occurs in one of the variants, increasing its frequency within the population, probably through interference mechanisms, a widely appreciated phenomenon of picornaviruses (164).

Other genetic mechanisms could be invoked to explain the evolution of progeny with an altered pathogenic spectrum, eg. genetic recombination or complementation. However, in view of the difficulties encountered in demonstrating genetic recombination between strains of picornaviruses and complementation between pairs of temperature-sensitive mutants (cf. Levintow, ref. 164), these mechanisms seem unlikely ones to explain the evolution of a strain of virus with altered biologic properties. Selection, and particularly mutation, are well described events (cf. Levintow, ref. 164). Indeed, the live attenuated polio vaccine is one such example (164,200), of a fortuitous mutation resulting in a virus with attenuated biologic properties.

The relative-permissive static-state model for Theiler's virus persistence in vivo suggests that some cells of the central nervous system are more permissive for Theiler's virus replication than others. The concept of different types of cells being more or less supportive of virus growth, is in itself, not unique and may be dependent on a variety of factors. A given cell population's relative permissiveness to support viral replication may be dependent on the ability of a cell within the population to produce interferon, or the susceptibility of the other cells in the population to the anti-viral effects of interferon. Failure of the cell to provide necessary enzymes, essential ions, amino acids, lipids, or other factors required for viral replication, may result in a reduction of viral maturation

compared to another cell capable of providing these factors. Although picornaviruses are notoriously lytic in susceptible cells, the amount of free virus produced during the infectious cycle, may vary depending on the host cell. The relative permissiveness of cells infected with picornaviruses may be dependent on the virus-host cell interactions at the viral transcriptional, translational or assembly level. If, for example, the regulatory mechanism which is responsible for maintenance of the ratio of plus to minus strands during the course of the infectious cycle was altered, ultimate progeny formation would be decreased. Deregulation of the proportion of plus RNA strands destined to serve as mRNA or genomic RNA may also serve to decrease progeny formation. Failure of the VPg protein to be covalently linked to the 5'-end of viral RNA may also decrease the quantity of virus produced in the infectious cycle. Failure of the virus to successfully inactivate eukaryotic initiation factors may result in a decrease of viral gene products at the translational level. The relative-permissive static-state model of Theiler's virus persistence would suggest that the neuron is the highly permissive cell type whereas other brain cell types, perhaps the oligodendrocyte, are less permissive; the relatively lower degree of permissiveness of these cells may involve one or more of these mechanisms.

Experimental Approach for these Studies:

The unusual biphasic disease course caused by Theiler's viruses, and the possibility that multiple variants of a single strain of the agent might exist in the central nervous system, provided the initial stimulus for the work presented in this study. It was believed that analysis of the biophysical, biochemical, and biologic properties of Theiler's virus isolated directly from acutely infected mouse brain, would not only provide defining criteria for the inclusion of Theiler's virus within the family Picornaviridae, but also provide evidence of whether or not multiple variants of the agents were present in acutely infected mouse brain. Moreover, by comparing the physiochemical properties of brain-derived virus with tissue culture adapted virus, it was hoped that biochemical markers could be elucidated which could be correlated with the disease states produced by brain-derived and tissue culture adapted viruses. These studies were based not on the premise that tissue culture adapted virus would bear genetic identity with any putative variant present in acutely infected mouse brain, but rather that comparison between tissue culture and brain-derived viruses would provide for some localization within the viral genome regions which may correlate with disease pathogenesis. Although Lipton, et. al. (176) compared the highly virulent with the less virulent strains of Theiler's viruses, and discovered molecular differences which were interpreted to correlate

with neurovirulence, this report describes the first biologic and biochemical analyses of a single strain of virus before and after tissue culture adaptation.

The WW strain of Theiler's virus was used throughout these studies because its pathogenesis has not been as well described in the literature as other strains of Theiler's virus. The experimental approach was to (1) establish histopathologically that the WW strain produced a biphasic disease course in mice similar to the well characterized biphasic disease produced by the DA strain of Theiler's virus, (2) to develop a method to purify virus directly from central nervous system tissues, (3) characterize some of the biophysical and biochemical properties of brain-derived virus, capable of producing both encephalomyelitis and chronic demyelinating diseases in vivo, (4) to adapt the WW strain to tissue culture, (5) to develop a method for purifying virus from tissue culture, (6) to characterize the biophysical and biochemical properties of the tissue culture adapted strain, (7) to assess the disease-inducing potential of tissue culture adapted virus and, (8) to compare biochemical and biophysical properties of tissue culture and brain-derived viruses to determine if biochemical markers existed which could be correlated with biologic properties observed in vivo.

The results reported here indicate that biochemical markers did exist, which could be used to distinguish tissue culture adapted from brain-derived virus preparations.

Furthermore, these results indicate that multiple variants of the WW strain of Theiler's murine encephalomyelitis virus exist in acutely infected mouse brain, and that one variant was lost during tissue culture adaptation; loss of this variant result in a viral preparation with attenuated encephalitogenic potential. The possible relationships between the results reported here and models of Theiler's persistence and chronic disease production are discussed.

MATERIALS AND METHODS

Brain-derived virus stocks (WW strain). Theiler's murine encephalomyelitis (TMEV) (WW strain) was obtained from Dr. D. Gilden (Philadelphia, PA). The virus had been passaged five times in suckling mice (ICR strain) by intracerebral inoculation of 0.02 ml of a 10% brain homogenate (348). Following receipt of TMEV(WW) as a 20% (w/v) brain homogenate of the fifth suckling mouse brain passage, the virus was passaged two times in suckling mice. Suckling mice were inoculated intracerebrally with 0.02 ml of clarified 10% (w/v) brain homogenate in the left hemisphere. Seven to nine days post-inoculation, brains were aseptically removed and homogenized in Hanks balanced salt solution using Ten Broeck tissue grinders to produce a 10% (w/v) homogenate. This stock contained 10^8 LD_{50}/ml as a clarified 10% (w/v) brain homogenate. When large quantities of virus were needed, suckling mice were inoculated intracerebrally with 0.02 ml of this seed stock containing 10^5 LD_{50} TMEV(WW). All brain homogenates were stored frozen at -70°C until used.

Preparation of tissue culture adapted virus (WW strain). Brain derived WW-TMEV was adapted to grow in BHK-21 (C-13) cells, a continuous line of baby hamster kidney cells; these cells were originally obtained from the American Type Culture Collection (Rockville, MD). Cells were propagated in Dulbecco's modified minimal essential media made to contain

10% (v/v) fetal calf serum, 100 u/ml of penicillin and streptomycin, 25 mM $MgCl_2$, 20.5 mg/ml tryptose phosphate broth (Difco Laboratory, Detroit, MI), 1 x minimal essential media vitamin solution (Microbiological Associates, Bethesda, MD) and 4.62 mg/ml bovine serum albumin, fraction V (Sigma Chemical Co., St. Louis, MO). Cells were maintained at 37°C in a 5% CO_2 humidified incubator. When the cells had reached confluence, they were switched to maintence media, which contained all of the above ingredients except fetal calf serum. When cells had reached maximum density they were trypsinized using a solution of trypsin (2.5%) in modified Hanks balanced salt solution without Ca^{+2} and Mg^{+2} (Microbiological Associates, Bethesda, MD). When sufficient cells had been grown, they were frozen at densities of 10^7-10^8/ml. For freezing, cells were trypsinized, concentrated by centrifugation at 400 x g for 10 min at 22°C. Cells were resuspended in a solution of Dulbecco's modified minimal essential media containing all of the supplements listed above, 20% fetal calf serum, and 10% dimethyl sulfoxide (DMSO). Cells were slow frozen to 70°C and then immersed in liquid nitrogen until needed. All experiments involving cell culture were performed using the same passage level of cells. For some experiments, cells were propagated in L-15 media (Microbiological Associates, Bethesda, MD) supplemented with 10% fetal calf serum, 2 mM glutamine and 100 U/ml penicillin and streptomycin. No differences in growth rates, morphology or ultimate cell densities achieved were

noted between Dulbecco's modified minimal essential media and L-15.

To prepare tissue culture adapted virus, confluent monolayers of cells were copiously washed with Hanks balanced salt solution (Biological Associates, Bethesda, MD) to remove all traces of fetal calf serum, and infected with TMEV(WW) as a clarified brain homogenate at a multiplicity of infection of 0.1-1 LD_{50}(suckling mouse doses) per cell. The virus was allowed to absorb to cells for 1 hr at 37°C. Following absorption, the viral inoculum was aspirated from the monolayers, and the monolayers washed with Hanks balanced salt solution. Media without fetal calf serum was added to the monolayer, and the monolayers returned to a CO_2 humidified 37°C incubator. Four days post-infection, although no evidence of cytopathic effect was observed, the monolayers were frozen at -70°C. The monolayers were put through three cycles of freezing and thawing, and following the last thawing cycle, tissue culture fluid was centrifuged at 400 x g to clarify it of cellular debris. The supernatants were then passaged onto new monolayers of cells, in a fashion identical to what was described above, at an unknown multiplicity of infection. This blind subpassage procedure was repeated 4 times. Upon the fourth passage, cytopathic effect was observed. The virus was passaged four more times <u>in vitro</u>, yielding a virus preparation which contained 10^6 pfu/ml. This stock of tissue culture adapted virus was used throughout these studies. When large quan-

tities of tissue culture adapted virus were needed, this seed stock was used to infect fresh cells at multiplicites of infection of 0.1-1 pfu/cell, and the progeny were harvested 4 days post infection as described above.

Another stock of tissue culture adapted WW-TMEV was a generous gift of Dr. H. Lipton (Chicago, IL). This stock had been passaged three times in BHK cells in his laboratory prior to receipt of the virus in this laboratory. Upon receipt the virus was passaged four times in BHK-21 (C-13) cells as described above.

Virus titration.

Titration of brain-derived stocks. Clarified, infected brain homogenates were made into serial 10-fold dilutions in Hanks balanced salt solution; the range of dilutions was from 10^0 (undiluted) to 10^{-9}. Groups of 10-20 ICR mice, less than 24 hours old, were inoculated intracranially with 0.02 ml of virus suspension per dilution. Animals were followed daily for the appearance of neurologic disease and death. The end point of the virus titration was defined as 18 day post inoculation. Virus titratations were calculated by the methods of Reed and Muench (265).

Titration of tissue culture adapted virus. Tissue culture adapted viruses were titered by both plaque assays and tissue culture infectious dose (TCID) assays. Titrations were carried out in 24-well plastic plates (Falcon). Virus to be titered was made into serial 10-fold dilutions in Hanks balanced salt solution and 0.1 ml added to each of

three replicate cells per dilution. The virus was allowed to absorb to infected cells for 1 hour at 37°C. At the end of the absorption period the viral inoculum was washed from the monolayers. For TCID assays, the cells were overlayed with media without calf serum. For plaque assays, the cells were overlayed with 1 ml of a solution of supplemented-Dulbecco's modified minimal essential media (see above) and 0.98% Difco Noble agar (Difco Laboratories, Detroit, MI). At three days post infection, plaque assay cultures were overlayed with 1 ml of neutral red in phosphate buffered saline at a concentration of 0.1 mg/ml. Virus titrations were defined to be complete at four days post infection for both plaque and TCID assays. The 50% endpoint of the virus titration by the TCID assay ($TCID_{50}$) was determined using the methods of Reed and Muench (265). The number of plaque forming units (pfu) per ml of the original tissue culture fluid was determined from the highest dilution of virus showing positive plaque formation; only those virus titrations exhibiting less than or equal to 10% variation between replicate samples were considered reliable.

Virus purification and quantitation.

Virus purification from infected brain homogenates. Supernatants of clarified brain homogenates were mixed with an equal volume of fluorocarbon (Freon^{R}-TF, DuPont, De-Memours, Co., Inc., Palo Alto, CA), vortexed for 10 min and centrifuged for 10 min at 400 x g. Brain lipids were extracted into the fluorocarbon (lower) phase; proteins and

virus remained in the aqueous (top) phase. The aqueous phase was carefully aspirated and subjected to fluorocarbon extraction three more times. The extracted virus was concentrated by pelleting for 1 hr at 280,000 x g in a Beckman ultracentrifuge with an SW41 rotor, (Beckman Instruments, Palo Alto, CA), resuspended in 0.02 M Trizma-HCl (Tris-HCl), pH 7.2, layered onto a preformed 15-35% (w/w) sucrose gradient and centrifuged for two hours at 280,000 x g. Approximate 0.4 ml fractions were collected and the OD^{280}, OD^{260} determined. Two peaks were obtained, the faster of which contained the maximum infectivity. The faster sedimenting peak fractions were pooled, adjusted to contain less than 10% (w/w) sucrose, pelleted for 2 hours at 280,000 x g, resuspended in CsCl with a density of 1.34 gm/cm^3 in 0.02 M Tris-HCl, pH 7.2, and centrifuged for 36 hours at 250,000 x g in a SW 50.1 rotor at 5^oC. Approximate 0.15 ml fractions were collected and those fractions containing virus pooled, adjusted to contain less than 7% CsCl and pelleted for 2 hours at 250,000 x g. The final pellet was resuspended in 0.02 M Tris-HCl, pH 7.2 and stored at -70^oC.

Purification of tissue culture adapted virus. Clarified tissue culture supernatants were further separated from cellular debris by centrifugation for 1 hour at 18,590 x g in a Beckman JA 20 rotor (Beckman Instruments, Palo Alto, CA). The supernatants were then centrifuged for 3 hours at 120,000 x g using an SW27 rotor to pellet the virus.

Pellets were resuspended in 0.02 M Tris-HCl, pH 7.2, and partially purified by centrifugation in sucrose gradients as described above. The fractions containing the virus were then concentrated by pelleting for 2 hr at 280,000 x g, resuspended and centrifuged in CsCl as above.

Quantitation of purified virus. Aliquots of purified virus were made into two serial 10-fold dilutions in 0.02 M Tris-HCl, pH 7.2 and the OD^{280}, OD^{260} determined. The quantity of virus present in the undilute aliquot of the purified virus preparation was determined empirically by the OD^{260}/OD^{280} ratio. For comparative experiments between tissue culture adapted and brain-derived viruses, the OD^{260}/OD^{280} ratios were adjusted so that each virus preparation contained the same ratio. Aliquots of virus preparations were checked for infectivity.

Sedimentation analysis. Sedimentation coefficients were calculated by the methods of McEwen from rate zonal sucrose gradient centrifugations, assuming a particle density of 1.30 gm/cm^3 (205). S values were derived for virus particles from CsCl gradients following removal of bound CsCl by repeated pelleting and washing steps for 2 hr at 280,000 x g in 0.02 M Tris-HCl, pH 7.2 prior to rate-zonal centrifugation in 5-15% (w/w) sucrose gradients. This step was required as some picornaviruses have been shown to bind CsCl; bound CsCl may alter sedimentation behavior (see Introduction).

Electron microscopy. Purified preparation of virus were examined by electron microscopy (EM) employing phosphotungstic acid (PTA), pH 6.0, as the negative stain. Two ul samples of virus were placed on polyvinyl formal filmed grids onto which was added a volume of PTA sufficient to cover the grid. Excess PTA was aspirated and the grid was allowed to dry at room temperature. EM was performed using a Philips model 300 electron microscope with acceleration voltages of 60 or 80 kV.

Radiolabeling of virus. Purified infectious virus was labeled in vitro using a modification of the chloramine-T method of Carthew and Martin (39). To ensure uniform labeling between different virus preparations, and allow for more accurate cross comparisons between brain-derived and tissue culture adapted preparations, the iodination procedure was standardized as follows. Aliquots of virus preparations were adjusted to contain identical OD^{260}:OD^{280} ratios per ml and iodinated using ratios of 3.9:1, chloramine-T: protein, 22 uCI ^{125}I:ug protein. The iodination reaction was allowed to proceed at room temperature for 2 min and stopped by addition of 10 ul of sodium metabisulphite at a concentration of 15 mg/ml. In other experiments, purified infectious virus was disrupted in 5% sodium dodecylsulfate (SDS) prior to radiolabeling by addition of an equal volume of 10% SDS to the virus preparation and boiling for 1 min. Labeled whole virus or disrupted virus was separated from unbound ^{125}I by gel filtration on a 3 ml

sephadex G-10 column equilibrated in 0.02 M Tris-HCl, pH 7.2, which had been previously exhaustively washed with 5% bovine serum albumin followed by 0.02 M Tris-HCl, pH 7.2 or 5% SDS in 0.02 M Tris-HCl, pH 7.2. 50 ul-fractions were collected. Whole virus preparations were also labeled with equal millimolar quantities of nonisotopic iodine. For some experiments, echovirus-12 was labeled in tissue culture. Four hours prior to infection, confluent monolayers of BSC-1 (Green monkey kidney) cells were treated with minimal essential media deficient in methionine. The cells were infected at multiplicities of infection of 0.01, and at the end of the 1 hr absorption period, the media was changed to minimal essential media containing 3 uCi/ml of ^{35}S methionine (Amersham-Searle, Arlington Heights, IL).

Polyacrylamide gel electrophoresis. Discontinuous sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on vertical 0.075 or 0.15 x 100 x 280 or 100 X 100 mm slabs using sample wells 10 mm wide formed in the stacking gel (159). The stacking gel prepared in 0.125 M Tris-HCl, pH 6.8 and 2 mM EDTA, consisted of the acrylamide monomer (5%, w/v) N, N'-methylene-bisacrylamide (Bis) (0.08% w/v), N, N, N', N'-tetramethylenediamine (TEMED) (0.05%, v/v), ammonium persulfate (0.1% w/v) and SDS (1.0%, w/v). The resolving gel prepared in 0.375 M Tris-HCl pH 8.9 and 2 mM EDTA, consisted of the acrylamide monomer (15%, w/v), Bis (0.4% w/v), TEMED (0.5%, v/v), ammonium persulfate (0.1%, w/v) and SDS (1.0% w/v). Immediately

prior to electrophoresis, samples of virus were processed by boiling for 1 min in sample buffer containing 67.4 mM Tris-HCl, pH 6.8, 1% SDS, 2 mM EDTA, 2% or 4% 2-mercaptoethanol (2-ME), 2% glycerol and 0.05% phenol red. For electrophoresis under non-reducing conditions, samples of virus were processed in sample buffer without 2-ME. Following preparation in the appropriate sample buffer, 50 ul of sample was loaded onto the gel and electrophoresed at a constant current of 14 mA using a model 220 or 221 electrophoresis unit (Bio-Rad Laboratories, Richmond, CA). Following electrophoresis, the gels were fixed at room temperature for 45 minutes in 12.5% (w/w) trichloroacetic acid, stained for 60-90 minutes in a solution of 0.2% Coomassie brillant blue R-250 (Sigma Chemical Co., St. Louis, MO) and destained at 50^{o}C overnight in 25% methanol and 7% glacial acetic acid, except in certain experiments outlined below. Gels were transferred to filter paper, dried under vacuum and exposed to Kodak XRP-1 or XR-1 Xray film at 22^{o}C or -22^{o}C or -70^{o}C. An EC transmission densitometer was used in some instances to locate polypeptides in autoradiograms.

Molecular weight determinations were performed by coelectrophoresing protein markers of known molecular weight in an adjacent well of the gel.

Re-electrophoresis of polypeptides. Certain experiments required isolation of viral polypeptide run under non-reducing conditions and re-electrophoresis under reducing conditions. Gels run under non-reducing conditions were

lightly fixed in 50% methanol, 10% acetic acid, and 1% Coomassie-blue for 30 minutes, destained for 60 minutes, sliced into 0.5 cm slices, and the radioactivity per slice determined. Slices containing polypeptides of interest were equilibrated for re-electrophoresis in 0.125 M Tris-HCl, pH 6.8, 1 mM EDTA and 0.1% SDS. Prior to re-electrophoresis the slices were boiled for 1 minute in reducing sample buffer containing 4% 2-ME. Slices were minced, placed in the sample well and the spaces around the pieces filled with 1% noble agarose in 0.125 M Tris-HCl, pH 6.8, 1 mM EDTA and 0.1% SDS. After addition of the running buffer the sample well was overlayed with 20 ul of the sample buffer in which the slice had been boiled. Following electrophoresis, the gel was processed as usual.

Limited proteolysis in gel. For certain experiments, polypeptides were subjected to peptide mapping by the limited proteolysis in gel method of Cleveland, et. al. (47). For these experiments, viral polypeptides of interest were located in gels following light fixation as described above. Slices containing the polypeptides of interest were re-equilibrated, boiled under reducing or nonreducing conditions, minced and prepared for electrophoresis as described above. Enzymatic digestion of the polypeptide was carried out by applying an overlay containing Staphylococcus Aureaus protease V8 (Miles Laboraties, Elkhart, ID) at a concentration of 1 ug/ul prepared in reducing or non-reducing sample buffer. Care was taken to ensure that di-

gestions occurred in enzyme excess. Current was applied to the electrophoresis unit, and when the phenol red tracking dye had reached and concentrated at the stacking gel:resolving gel interface, the current was turned off for 30 min. Following the 30 minunte digestion period, the current was reapplied and electrophoresis continued. Following electrophoresis, the gel was processed as usual.

Trypsin digestion of whole virus. Equivalent preparations of whole radiolabeled TMEV(WW) were prepared. One aliquot was treated for 20 min at 37°C with 100 ug of trypsin in 0.02 M Tris-HCl buffer, pH 7.2. Control aliquots containing equivalent counts per minute were incubated with an equivalent amount of buffer without trypsin. Following digestion, the samples were subjected to electrophoresis under non-reducing and reducing condition as described above.

Viral RNA characterization.

Extraction of RNA from purified virions. Aliquots of purified brain-derived virus were treated with 200 ug of proteinase-K and 1% SDS at 56°C for 1 hr. To 200 ul of the protease treated virus suspension, 300 ul of SDS-citric acid-sodium phosphate (SDS-CP) buffer was added (0.1 M citric acid, 0.1 M disodium phosphate, 1 mM EDTA, 0.2 M SDS, 0.1% diethyl pyrocarbonate (DEPC), pH 4.1) and incubated at 37°C for 30 min. The extracted RNA was centrifuged through 15-30% (w/w) sucrose gradients in SDS-TNE buffer (0.5% SDS, 0.1 M NaCl, 0.02 M Tris-HCl, pH 7.5, 1 mM EDTA, 0.1% DEPC)

at 100,000 x g for 10.5 hr at 22°C. The gradient was fractionated into approximately 20 fractions and the OD^{260}, OD^{280} determined. Those fractions containing the highest OD^{260}:OD^{280} ratio, were pooled and the RNA precipitated at -20°C for 18 hours by addition of sodium acetate and absolute ethanol at final concentrations of 0.4 M sodium acetate and 70% ethanol. The RNA precipitate was pelleted for 5 min in a Beckman microfuge centrifuge (Beckman Instruments, Palo Alto, CA), dried under vacuum and resuspended in 0.5 ml of 5 mM EDTA, 0.2% DEPC, pH 7.0.

Further removal of protein from the extracted RNA was accomplished by phenol extraction. The RNA was re-ethanol precipitated and adjusted to contain 0.1 M sodium acetate and 0.5-1% SDS. An equivalent volume of redistilled phenol, saturated with TNE buffer was added and the solution vortexed at room temperature for 10 minutes. An equivalent volume of chloroform was added and the mixture centifuged in the Beckman microfuge at room temperature for 2 minutes. The chloroform-phenol extraction was repeated three times. The aqueous phase was adjusted to contain 0.4 M sodium acetate and 70% ethanol and left at -20° C until frozen. The solution was then thawed slighly and then centrifuged in the microfuge to pellet the precipitated RNA. The ethanol precipitation step was repeated three times. The final pellet was resuspended in 5 mM EDTA and the OD^{260} determined. The concentration of RNA was based on the value of 43 ug RNA is equivalent to 1 OD^{260}. The RNA solution was

then adjusted to contain 70% ethanol and stored at -70°C until used.

Preparation of antisera. Antibodies to brain-derived WW-TMEV were produced in rabbits. New Zealand white rabbits were inoculated intracranially with $10^5 LD_{50}$ (suckling mouse dose) brain-derived WW-TMEV. A control rabbit was simultaneously inoculated with uninfected suckling mouse brain homogenate. Three months later, the rabbit inoculated with infected brain homogenate was inoculated with $10^9 LD_{50}$ purified TMEV(WW) intravenously, and subcutaneously along the back with $10^{9.8} LD_{50}$ mixed with complete Freund's adjuvant. Subcutaneous inoculation of purified virus in complete Freund's adjuvant was repeated one month later. Throughout the sensitization procedure, the animal was bled, the serum separated from red blood cells, and stored at -70°C. Pre-immune serum was also obtained.

To remove non-specific reactivity from immune serum, immune sera were adsorbed against uninfected mouse brains. The immune serum reached a maximum titer of 1:256 one month after the last sensitization with purified virus in complete Freund's adjuvant, as measured by hemagglutination inhibition, using human type O red blood cells (176). This increase in titer represented an increase of 2^5 over pre-immune serum from the same animal. For most experiments, the sera were heat-inactivated (56°C for 30 min) before use.

Immunofluorescence. For some experiments, infected brains were removed, snap frozen, and sectioned into 10

micron cross sections using an AO cryostat. For other experiments, infected and control cell monolayers grown on cover slips, were used in immunofluorescent studies. In both cases, materials for immunofluorescent studies were air dried, fixed in acetone, washed in phosphate buffered saline, and overlayed with WW-TMEV antiserum prepared in rabbits or commercially prepared mouse anti-GDVII serum (Microbiologic Associates, Bethesda, MD) for 20 min at room temperature in a humidified chamber. Monolayers were then washed in phosphate buffered saline 2 times for 5 min each, and overlayed with the appropriate fluorescein conjugated anti-globulin serum for 20 min at room temperature in the humidified chamber. All anti-globulin fluorescein conjugated sera were obtained from Cappel Laboratories, Inc. (Cappel Lab., Inc., Cochranville, PA). Following the final incubation, the specimens were placed on glass slides in neutral glycerol and observed with a Dialux-20 epi-illumination Leitz fluorescent microscope. Controls consisted of uninfected monolayers or tissues as well as infected tissues or monolayers which were treated with one antiviral serum (eg. rabbit) followed by another viral antisera (eg. mouse) and then a fluorescein conjugated antiglobulin directed to the second immunoglobulin (mouse). Positive fluorescence was blocked due to the presence of the primary antiserum (rabbit).

Immunoprecipitation studies. Some experiments required determination of the immunoreactivity of individual polypeptides. Preparations of radiolabeled virus were

electrophoresed (see above), and at the end of the run, the gel was fixed. The fixed gel was sliced into 0.25 or 0.5 cm slices. Each slice was placed in 1 ml of barbital-triton buffer (0.1 M sodium barbital, pH 8.9, 0.1% triton-X-100). Slices were placed on a rotary shaker at 37°C for 24 hr. The supernatant buffer solution was removed and the radioactivity extracted from each slice determined. Aliquots of the radioactive extracted proteins were adjusted to contain equivalent amounts of radioactivity and the total volume of the reaction mixture adjusted to 50 ul. Preparations of whole ^{125}I-labelled virus, adjusted to contain the same percent barbital, triton X-100 and SDS served as controls. All samples received 50 ul of rabbit anti-WW-TMEV serum, rabbit pre-immune serum, mouse anti-GDVII serum (see "Preparation of antisera" above), commercially prepared normal rabbit serum (Microbiological Assoc., Bethesda, MD), or rabbit serum from a rabbit inoculated with normal mouse brain homogenates. All samples of viral proteins treated with each antibody were done in triplicate. The samples were incubated at 37°C for 40 min-60 min. Other controls consisted of whole virus or viral protein eluates incubated in the absence of any serum. 100 ul of Staphylococcus aureus protein A (Pansorbin^{R}, Cal. Biochem.-Behring Corp., San Diego, CA) were added to all samples and placed at 4°C for 30 min to bind immune complexes. The Pansorbin^{R} and immune complexes were pelleted by centrifugation in a Beckman Microfuge for 4.5 min. Supernatants were removed and

pellets resuspended and washed in barbital-triton buffer and repelleted. The washing steps were repeated an average of 4-5 times, until stable quantities of radioactivity were achieved in the supernatant and pellet fractions. The calculations of specific immunoreactivity were determined by comparing the specific reactive index ($\bar{X}$% CPM in pellet of virus or protein + antibody/$\bar{X}$% CPM in pellet of virus or protein + NRS) to the non-specific reactive index ($\bar{X}$% CPM in pellet of virus or protein + serum/$\bar{X}$% CPM in pellet of virus or protein - serum). Specific reactive indices of virus proteins were then compared with the specific reactive indices of whole virus.

In other experiments, the Pansorbin^{R}-immune complexes were disrupted in SDS-PAGE sample buffer by boiling for 5 min and vigorous sonication (Branson Model W350 sonicator). Unsolubilized components were removed from the supernatant by pelleting in the Beckman Microfuge for 4.5 min. All reactions were performed in antibody excess. Samples for electrophoresis were loaded directly, regardless of the specific radioactivity per sample. This procedure was adopted to insure that an adequate assessment of the specific reactivity of the immunoglobulin present in each sample sera would be properly assessed. Following electrophoresis the gel was processed for autoradiography and the results quantitated by scanning densitometry.

Histology. Infected and control animals were perfused with normal saline or phosphate buffered saline followed by

Bouin's fixative, 10% buffered formalin, or a solution containing 75% ethanol and 25% glacial acetic acid. The tissues were blocked, embedded in paraffin, sectioned into 6 micron sections and stained for routine histologic examination in hematoxylin-eosin (H & E), or for myelin by Weil or Luxol fast blue stains, or for nerve processes and axons, using a Bodian stain.

RESULTS

I. Histopathology and Clinical Course Following Inoculation with Brain-Derived Theiler's Virus (WW Strain).

Approximately 7-9 days post intracerebral (IC) inoculation of weanling mice with 10^3 LD_{50} (suckling mouse doses) of brain-derived WW-TMEV ((B-TMEV (WW)), mice began to develop hind limb paresis, flaccid paralysis, or encephalitis. Signs which indicated encephalitis included lethargy, ruffled fur, cachexia, or circling behavior. Histologically, this acute phase was characterized by chromatolysis of neurons, neuronophagia, and microglial proliferation. This was most most pronounced in the hippocampus and anterior horns of the spinal cord (Fig. 5). Approximately 20-30% of inoculated animals survived the acute infection and began to develop the chronic phase of disease approximately 30-40 days later, exhibited by a mild hind limb plegia. Only in those animals which did not exhibit minor residual paralysis from the acute phase, could the modest gait disturbance be detected.

This phase of disease was characterized histologically by focal collections of mononuclear cells in spinal cord leptomeninges and white matter. The inflammatory response continued throughout the infection, and by 90 days post inoculation, severe demyelination of primarily the ventral and lateral columns of the spinal cord whiter matter was easily detected (Fig. 6).

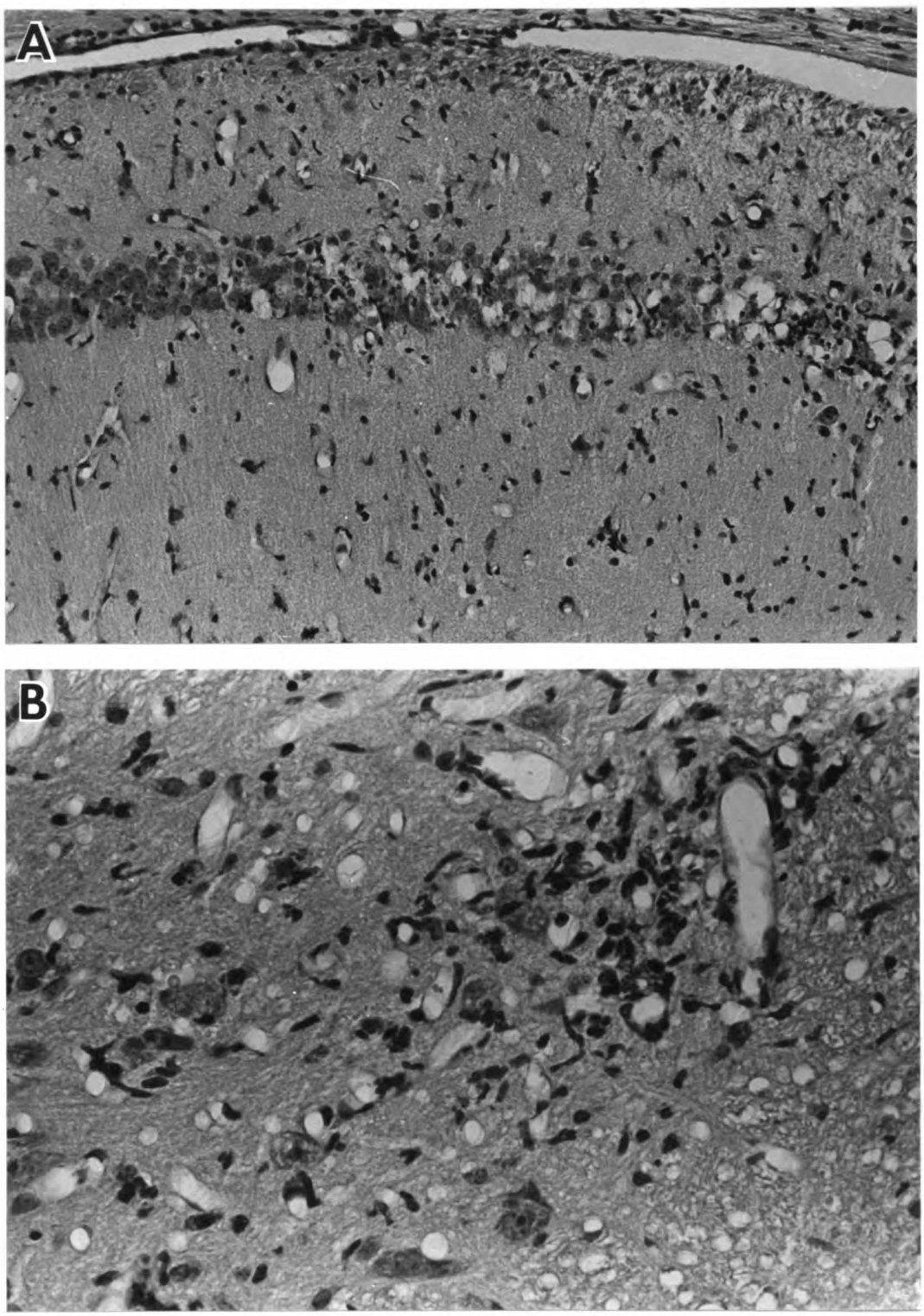
Legend to Figure 5

HISTOPATHOLOGY OF THE ACUTE PHASE OF DISEASE IN THE CENTRAL NERVOUS SYSTEM OF WEANLING MICE FOLLOWING INTRACRANIAL INOCULATION WITH BRAIN-DERIVED THEILER'S VIRUS (WW STRAIN).

(A) Coronal section through the hippocampus of a mouse inoculated with brain-derived Theiler's virus (WW strain) and sacrificed at 13 days post-inoculation. Numerous microglia and mononuclear cells are present in the hippocampal fissure (bottom). Hippocampal neurons have undergone chromatolysis and are vacuolated while others are surrounded by microglia and inflammatory cells (neuronophagia). (H & E, magnification, 100X)

(B) Cross-section through the spinal cord of the mouse shown in (A). Note the severe neuronophagia of anterior horn cells by microglia and the presence of numerous mononuclear cells. The white matter (lower right) is free of inflammatory cells. (H & E, magnification, 250X).

FIGURE 5



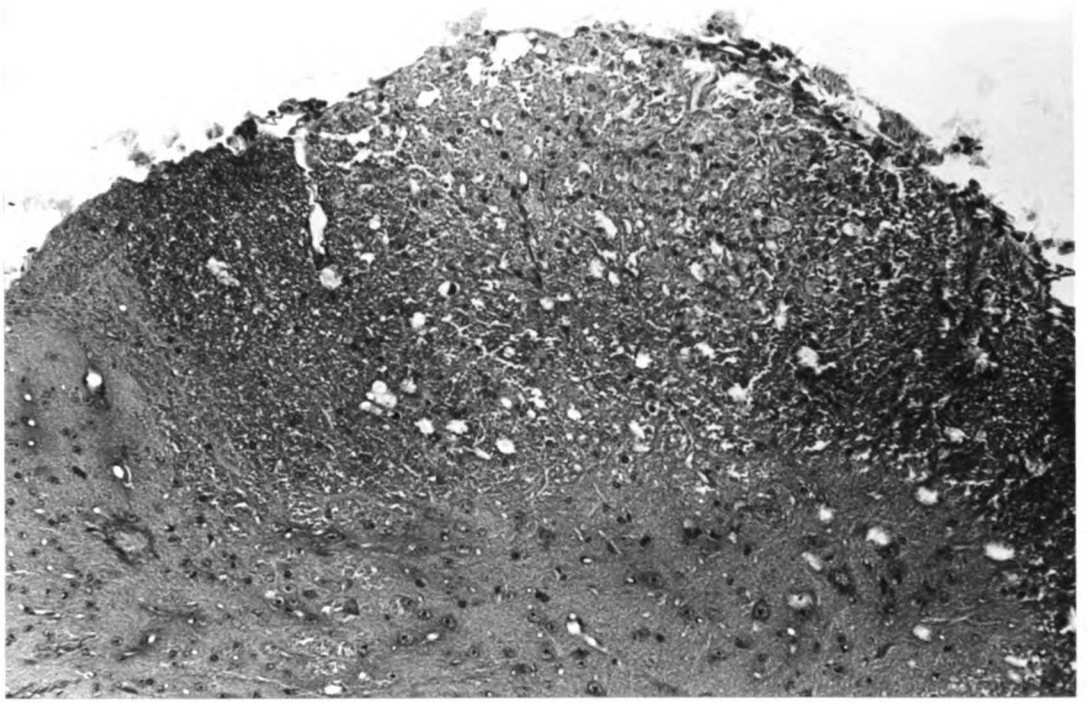


Figure 6

HISTOPATHOLOGY OF THE CHRONIC PHASE OF DISEASE IN THE CENTRAL NERVOUS SYSTEM OF WEANLING MICE FOLLOWING INTRACRANIAL INOCULATION OF BRAIN-DERIVED THEILER'S VIRUS (WW STRAIN).

Cross-section of a spinal cord of an animal inoculated 90 days before sacrifice with brain-derived Theiler's virus (WW Strain). A large demyelinated lesion can be seen in the lateral columns (top). Some mononuclear cells can be seen in the lesion and leptomeninges. The gray matter (bottom) is uninvolved. (Weil stain, magnification, 100X).

When similar studies were performed using suckling mice, less than 24 hours old at the time of inoculation, essentially similar results were obtained, with the exception that lysis of neurons was more extensive. By 11 days post inoculation severe chromatolysis of neurons could easily be detected in the cortex, brainstem, nuclei, and most notably, in the stratum pyramadale hippocampi of the hippocampus (Fig. 7A). Furthermore, few suckling mice inoculated IC with even low doses of brain derived virus (eg. 1 x $10^{1.7}$ LD_{50}) survived the acute phase of disease. In contrast, as many as 50% of weanling mice, 19-21 days old, survived IC inoculation of 10^3 LD_{50} (suckling mouse doses) and developed the chronic phase of disease.

During the acute phase of infection, mice inoculated as weanlings were found to contain between 10^7-10^8 LD_{50}/gram of central nervous system (CNS) tissues of B-TMEV(WW). During the chronic phase of infection mice were found to contain between 10^1-10^2 LD_{50}/gm CNS tissue of infectious virus through 186 days post inoculation. It was found in 2 animals taken at 186 days post inoculation, that spinal cord contained ten times more per gram of the infectious virus than brain.

Viral specific immunofluorescence was observed in brains of both suckling and weanling animals during the acute phase of infection (Fig. 7B). Specific fluorescence could be observed in hippocampus, cortex, anterior horns of

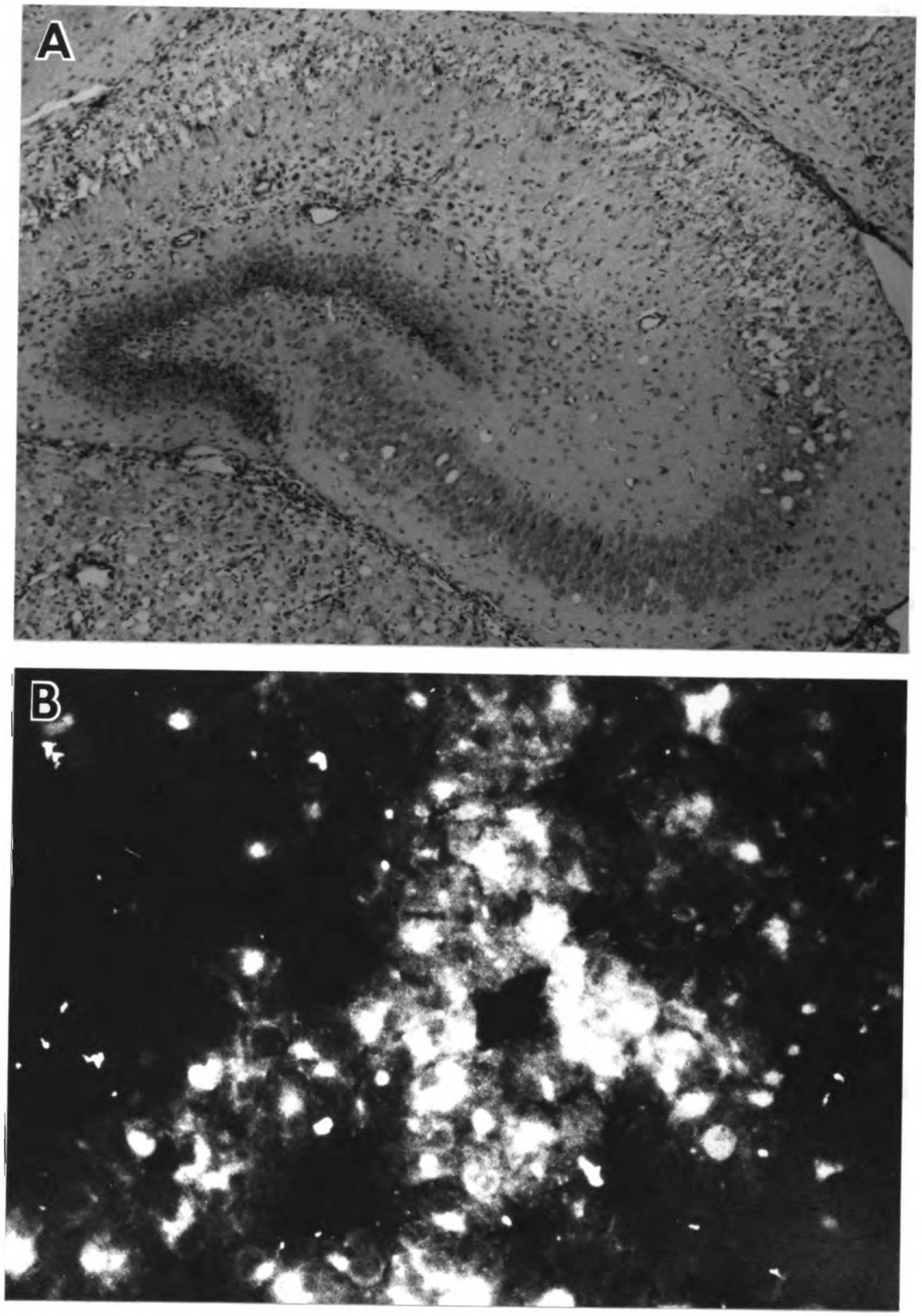
Legend to Figure 7

HISTOPATHOLOGY OF THE ACUTE PHASE OF DISEASE IN THE CENTRAL NERVOUS SYSTEM OF SUCKLING MICE FOLLOWING INTRACRANIAL INOCULATION WITH BRAIN-DERIVED THEILER'S VIRUS (WW STRAIN).

(A) Coronal section of the hippocampus of a mouse inoculated with brain-derived Theiler's virus (WW strain) and sacrificed at 11 days post-inoculation. Severe chromatolysis of the stratum pyramadale hippocampi of the hippocampus can be seen. Vessels in the hippocampal fissure are cuffed by mononuclear cells. (H & E, magnification 25X).

(B) Theiler's virus (WW strain) specific immunofluorescence in the cortex of an animal inoculated and sacrificed as (A) above. The brain was frozen in liquid nitrogen, sectioned into 15 micron sections, and stained for viral antigens with Theiler's virus (WW strain) specific antiserum and an appropriate fluorescein conjugated antiglobulin. (magnification, 250X).

FIGURE 7



the spinal cord, and occasionally brainstem. No convincing viral specific immunofluorescence could be observed in any CNS tissue during the chronic phase of infection.

No incidence of clinical disease was observed in weanling mice following intraperitoneal inoculation of $10^{1.7}$-$10^{5.7}$ LD_{50} (suckling mouse doses determined by IC inoculation) of B-TMEV(WW).

II. Tissue Culture Adaptation of Brain-Derived Theiler's Virus (WW Strain). Stocks of B-TMEV(WW) containing between 10^{1} - 10^{8} LD_{50}/ml of infectious virus (suckling mouse doses) were found to be unable to produce cytopathic effect (CPE) when inoculated as clarified homogenates onto primary cell cultures, including ICR baby mouse kidney cultures, suckling or weanling brain cultures, cynomogolus monkey kidney cultures, Rhesus monkey kidney cultures and when infected brains were cocultivated with any of these cells. These experiments were performed using media containing between 1 and 5% fetal calf serum (FCS) and gammaglobulin free FCS, or in the presence of FCS plus SV_5 antiserum. Therefore, a blind-subpassage method of tissue culture adaptation (169) was performed using baby hamster kidney fibroblasts (BHK-21 (C-13)). Adaptation was defined to be complete with the production of CPE, and adaptation was achieved after the fourth subpassage.

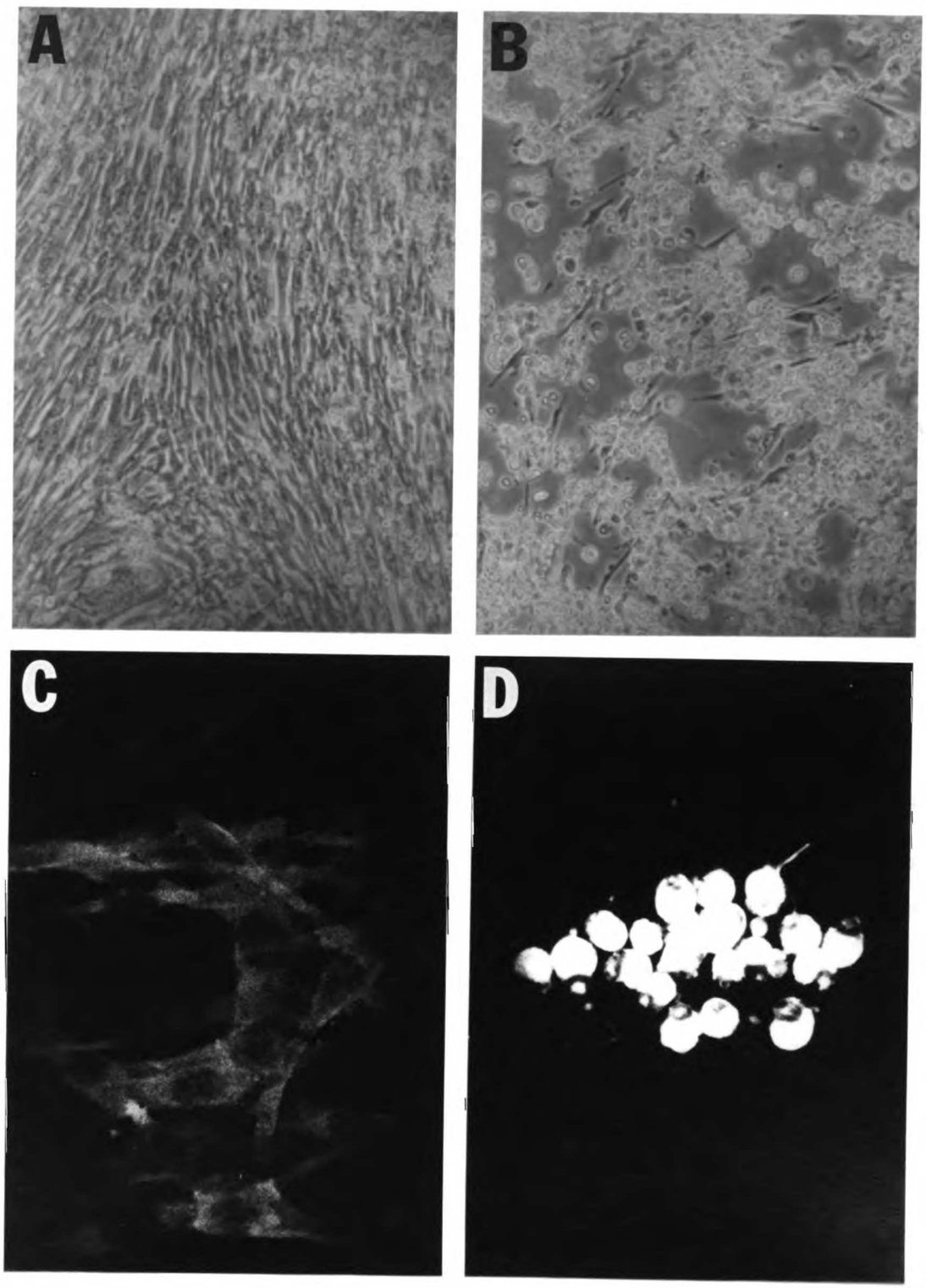
Figure 8 illustrates the typical picornaviral CPE produced by the virus following adaptation. Immunofluorescence studies using rabbit hyperimmune antisera or hetero-

Legend to Figure 8

CYTOPATHIC EFFECT OF TISSUE CULTURE ADAPTED THEILER'S VIRUS (WW STRAIN) ON BHK-21 (C-13) CELLS.

Monolayers of BHK-21 (C-13) cells were infected with 10^5 pfu tissue culture adapted Theiler's virus (WW strain) (B,D) or uninfected tissue culture supernatant (A,C) and photographed 3 days after infection. Control (C) and infected (D) cells were examined for presence of Theiler's virus (WW strain) antigens by immunofluorescence using specific antisera and an appropriate fluorescein isothiocyanate conjugated anti-globulin. Intensely staining viral specific inclusions can be seen in infected cells (D) which occupy most of the cytoplasm. (A,B. magnification, 40X; C,D, magnification, 250X)

FIGURE 8



typic antisera confirmed the presence of viral antigen in infected cells (Fig. 8). When tissue culture adapted TMEV(WW) obtained from H. Lipton was grown in BHK-21 (C-13) cells at the same input multiplicity of infection, the same type and temporal appearance of CPE occured as with tissue culture adapted TMEV(WW).

III. Histopathology and Clinical Course Following Inoculation of Tissue Culture Adapted Viruses. IC inoculation of mice with $10^{4.3}$ pfu of tissue culture adapted WW-TMEV (TC-TMEV(WW)) did not result in any easily detectable signs until 14 days post inoculation, when approximately 1% of infected animals exhibited a slight hind limb gait disturbance reminiscent of the gait of mice chronically infected with B-TMEV(WW). Also in contrast to B-TMEV(WW) infection, histologic lesions appeared much later, and were predominately found in white matter only. Between 7-11 days post inoculation, very few if any inflammatory cells could be detected in the hippocampus, thalamus, brainstem, or cortex, and no anterior horns of spinal cord exhibited inflammatory cells (Fig. 9). The lesions first became easily detectable at 30-40 days post-inoculation and appeared to become larger throughout the course of infection. Lesions could be detected through 78 days post inoculation (Fig. 9).

These observations confirmed other studies (62, 175) that the major biological difference between B- and TC-TMEV(WW) was that only B-TMEV(WW) was capable of inducing encephalomyelitis, that TC-TMEV(WW) produced clinical signs

Legend to Figure 9

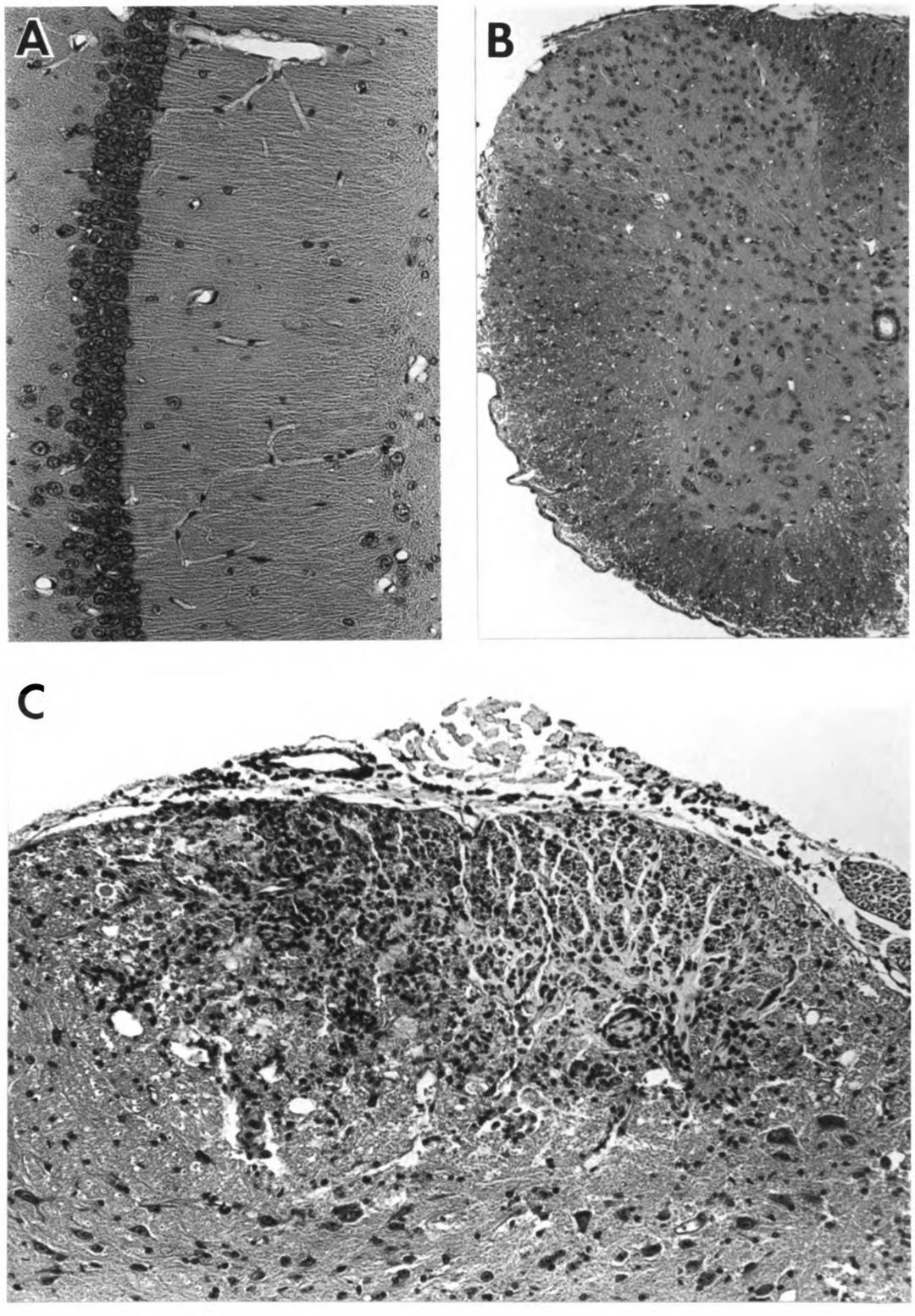
HISTOPATHOLOGY IN THE CENTRAL NERVOUS SYSTEM OF WEANLING MICE FOLLOWING INTRACRANIAL INOCULATION WITH TISSUE CULTURE ADAPTED THEILER'S VIRUS (WW STRAIN).

(A) Coronal section through the hippocampus of a mouse inoculated with tissue culture adapted Theiler's virus (WW strain) and sacrificed at 11 days post-inoculation. Few inflammatory cells can be hippocampal fissure (right), and no degenerative changes in the neuron of the stratum pyramadale hippocampi (left). (H & E, magnification, 100X)

(B) Cross-section through the spinal cord of the mouse shown in (A). No inflammatory response can be observed in the gray or white matter of the spinal cord. (H & E, magnification, 63X)

(C) Cross-section through the spinal cord of a mouse inoculated with tissue culture adapted Theiler's virus (WW strain) 78 days before sacrifice. A large inflammatory lesion can be seen extending from the leptomeninges through the lateral columns of the spinal cord white matter (top). An apparent area of gliosis is visible in the center of the lesion. The gray matter (bottom) is preserved. (H & E, magnification, 100X)

FIGURE 9



and histologic lesions later in infection than B-TMEV(WW), and that the lesion distribution of TC-TMEV(WW) was restricted primarily to white matter of the spinal cord.

IV. Viral Isolation and Physical Characterization.

When crude extracts of B-TMEV(WW) were pelleted and centrifuged through linear 15-35% (w/w) sucrose gradients, two peaks were obtained (Fig. 10B) the faster of which was not present in uninfected brain homogenate similarly treated (Fig. 10A). IC inoculation of an aliquot of peak I (Fig. 10B) into suckling mice resulted in encephalitis, where as an aliquot of peak II (Fig. 10B), adjusted to contain the same percent of sucrose as peak I did not. When preparations of TC-TMEV(WW) were centrifuged through linear sucrose gradients similar results were obtained (data not shown).

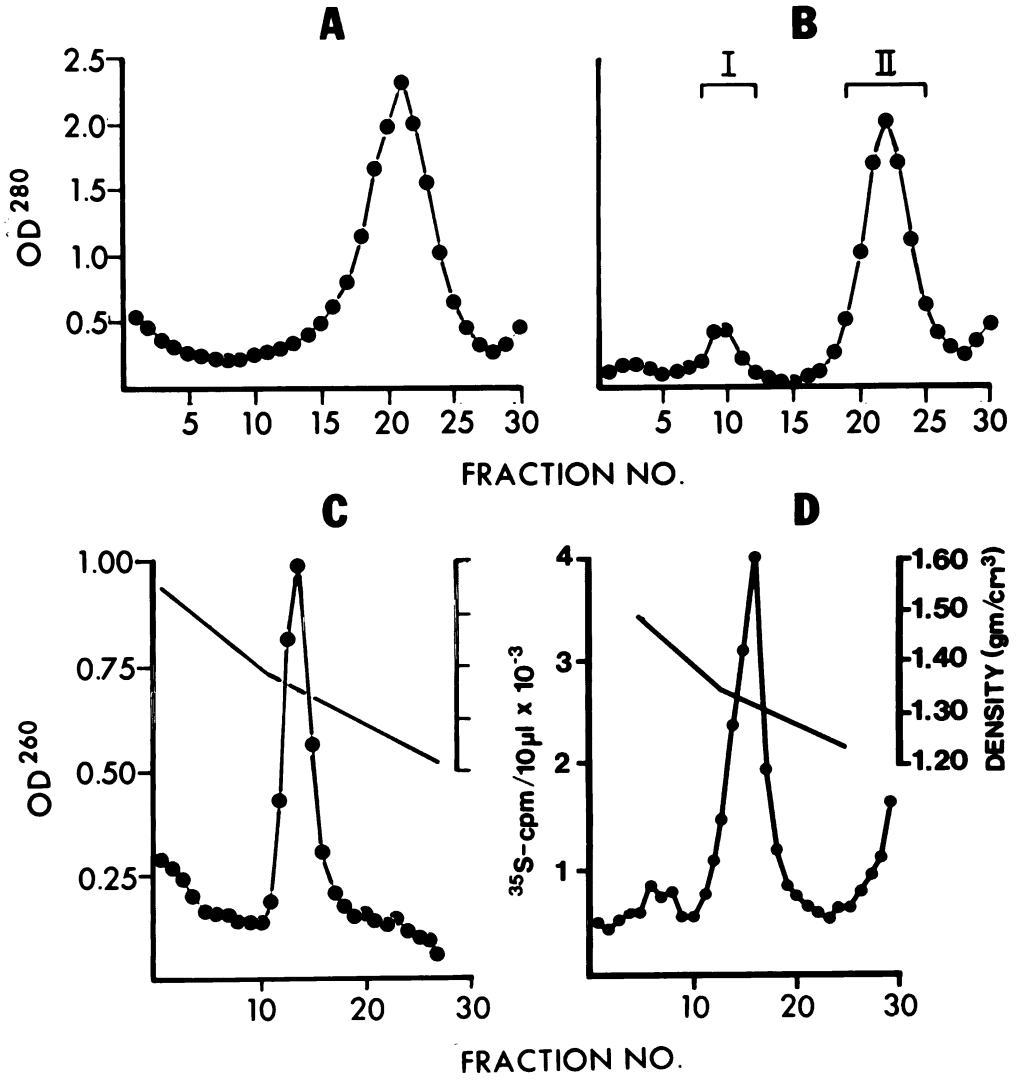
The faster sedimenting fractions of peak I (Fig. 10B) of B- and TC-TMEV(WW) were pooled, pelleted and centrifuged in CsCl. B-TMEV(WW) (Fig. 10C) and TC-TMEV(WW) (data not shown) banded at a buoyant density of 1.35 gm/cm^3. Rate-zonal centrifugation following removal of bound CsCl revealed these particles to have an estimated sedimentation coefficient of 156 ($S_{20}\omega$). In comparison, echovirus-12 banded in CsCl at a buoyant density of 1.33-1.34 gm/cm^3 (Fig. 10D) and had a sedimentation coefficient of 159 ($S_{20}\omega$) in the rate-zonal sucrose gradient. The modest differences in sedimentation coefficients were not considered significant. B-TMEV(WW) from the CsCl gradient was

Legend to Figure 10

SUCROSE AND CESIUM CHLORIDE DENSITY CENTRIFUGATION OF THEILER'S VIRUS (WW STRAIN) AND ECHOVIRUS-12.

Sucrose density gradients of clarified uninfected (A) and Theiler's virus (WW strain) infected ICR mouse brain homogenates (B). Suckling mice were inoculated with equal volumes of infected and uninfected brain homogenate. At 9 days post-infection, brain homogenates were treated with fluorocarbon as described in Materials and Methods. Supernatants were centrifuged through 15-35% sucrose gradients for 2 hr at 280,000 x g. Centrifugation was from right to left. Peak I (B) contained virus and was further purified by density gradient centrifugation in CsCl for 36 hr at 250,000 x g (C). Infectious virus banded at 1.35 gm/cm^3. Echovirus-12 was radiolabeled with ^{35}S-methionine in tissue culture, harvested and purified by rate-zonal sucrose gradient centrifugation followed by equilibrium density centrifugation in CsCl. Infectious virus banded at 1.33-1.34 gm/cm^3 in CsCl (D).

FIGURE 10


A
B
I
II
2.5
2.0
1.5
1.0
0.5
OD 280
5 10 15 20 25 30
FRACTION NO.
C
D
1.00
0.75
0.50
0.25
OD 260
4
3
2
1
35S-cpm/10µl x 10-3
1.60
1.50
1.40
1.30
1.20
DENSITY (gm/cm3)
10 20 30
FRACTION NO.

found to be highly infectious when inoculated IC into suckling mice, and consisted of a homogeneous population of intact 26 nm nonenveloped particles by electron microscopy (EM) (Fig. 11). It was not possible to appreciate the icosahedral symmetry by these techniques. TC-TMEV(WW) was also found to be highly infectious when inoculated onto monolayers of BHK-21 (C-13) cells, and had the same size as B-TMEV (WW) by EM (data not shown).

A smaller peak with a density of 1.27 gm/cm^3 was occasionally present in preparations of both B- and TC-TMEV(WW) and was found to not be infectious as measured by an inability to produce either acute TMEV disease following IC inoculation in suckling mice (B-TMEV(WW)), or its failure to produce CPE (TC-TMEV(WW)). By EM this material did not contain intact virions and was presumably artifical top component (ATC), an artifact seen in some CsCl gradients of picornaviruses (see Introduction, Table 7).

These results indicated that there were no major differences in the biophysical characteristics of B- and TC-TMEV(WW). Additionally, they offered firm evidence that these strains of Theiler's virus had biophysical characteristics in common with other picornaviruses (see Introduction, Table 6).

V. Partial Characterization of the Nucleic Acid of Brain-Derived Theiler's Virus. Although the nucleic acid of Theiler's viruses is known to be RNA (89), the biohysical characteristics of the RNA have not been reported. SDS-

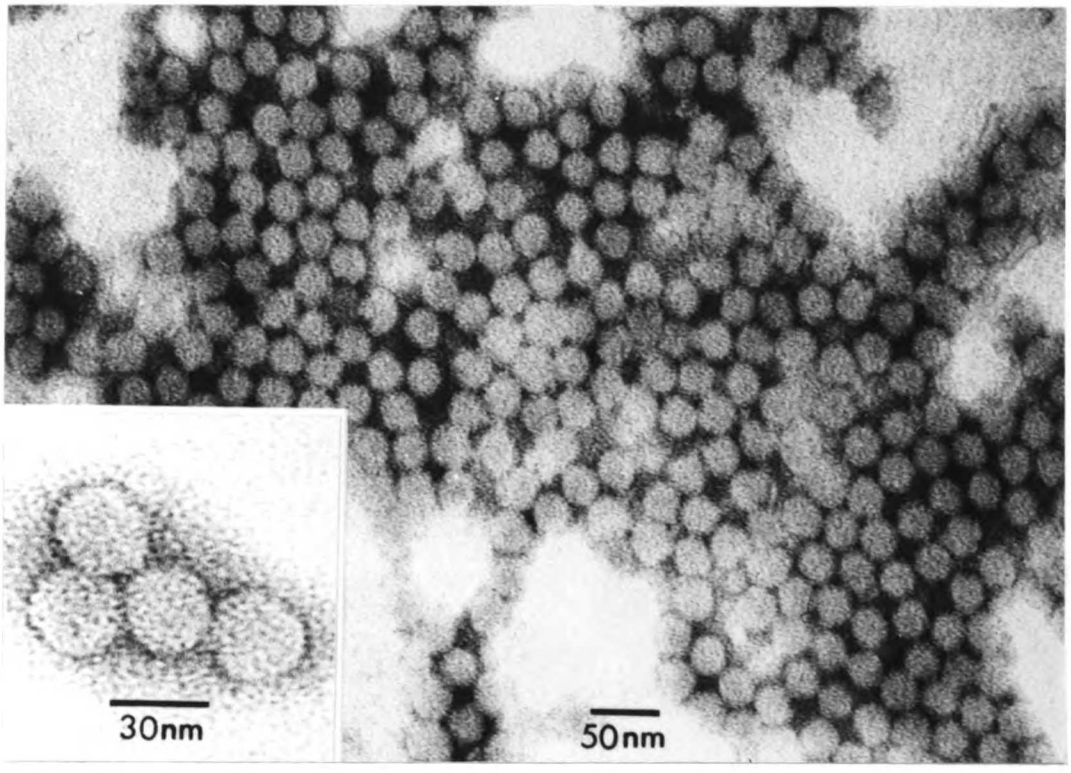


Figure 11

NEGATIVE STAIN OF PURIFIED BRAIN-DERIVED THEILER'S VIRUS (WW STRAIN).

Theiler's virus (WW strain) was extracted by fluorocarbon from infected ICR mouse brain, and purified by sucrose and CsCl density gradient centrifugation (see legend to Figure 10). Two-ul of purified virus was negatively stained with 2% PTA, and examined by electron microscopy prior to SDS-PAGE.

citric acid extraction of RNA from purified preparations of B-TMEV(WW) were performed following solubilization and digestion of the viral capsid in proteinase-K and SDS. The extracted RNA was centrifuged through linear 15-30% (w/w) rate-zonal sucrose gradients to separate it from contaminating protein. Following gradient fractionation, those fractions containing the RNA were precipitated and further removal any contaminating protein from extracted RNA was accomplished by phenol extraction. Rate-zonal centrifugation was repeated on an aliquot of the purified RNA to determine its sedimentation coefficient with respect to ^{3}H-rRNA centrifuged in an identical sucrose gradient at the same time (Fig. 12). The viral RNA had an estimated sedimentation coefficient of 35S in common with other members of the Picornaviridae (see Introduction, Table 6).

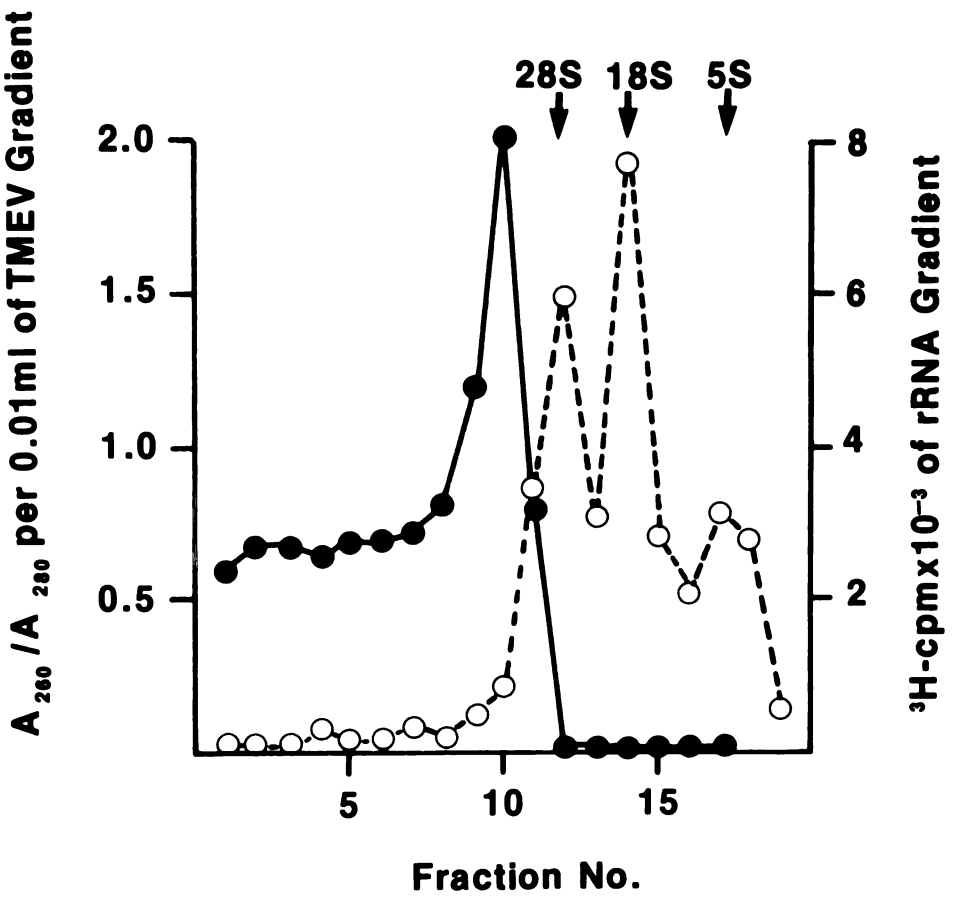
VI. Polypeptides of Theiler's Virus (WW Strain). Because of the inherent inefficiency and the poor reliability of labeling B-TMEV(WW) by radiolabeled amino acid incorporation in vivo, methods were developed whereby both brain-derived and tissue culture adapted viral polypeptides could be prepared and detected under identical conditions. It was believed important to radiolabel both B- and TC-TMEV(WW) under identical conditions so that accurate comparisons could be made, without the introduction of variability due to the use of different techniques (e.g. radiolabeling in tissue culture vis a vis radiolabeling in vitro).

Legend to Figure 12

RATE-ZONAL SUCROSE DENSITY CENTRIFUGATION OF THEILER'S VIRUS (WW STRAIN) RNA.

RNA was extracted from infectious brain-derived Theiler's virus (WW strain) in SDS-citric acid following solubilization of the capsid in proteinase-K and SDS and removal of contaminating protein by chloroform-phenol extraction. Extracted viral RNA was centrifuged through 15-30% (w/w) sucrose gradient in SDS-TNE buffer for 10.5 hr at 100,000 x g (●———●). ^{3}H-rRNA was co-centrifuged in an identical sucrose gradient (O– – –O). The gradients were fractionated, and the measured refractive indices were used to adjust the gradients with respect to each other. The positions of the 28S, 18S, and 5S rRNA subunits are indicated. Viral RNA had an estimated sedimentation coefficient of 35S relative to the rRNA markers.

FIGURE 12


28S
18S
5S
2.0
1.5
1.0
0.5
8
6
4
2
5
10
15
A_{280}/A_{280} per 0.01ml of TMEV Gradient
^{3}H-cpmx10^{-3} of rRNA Gradient
Fraction No.

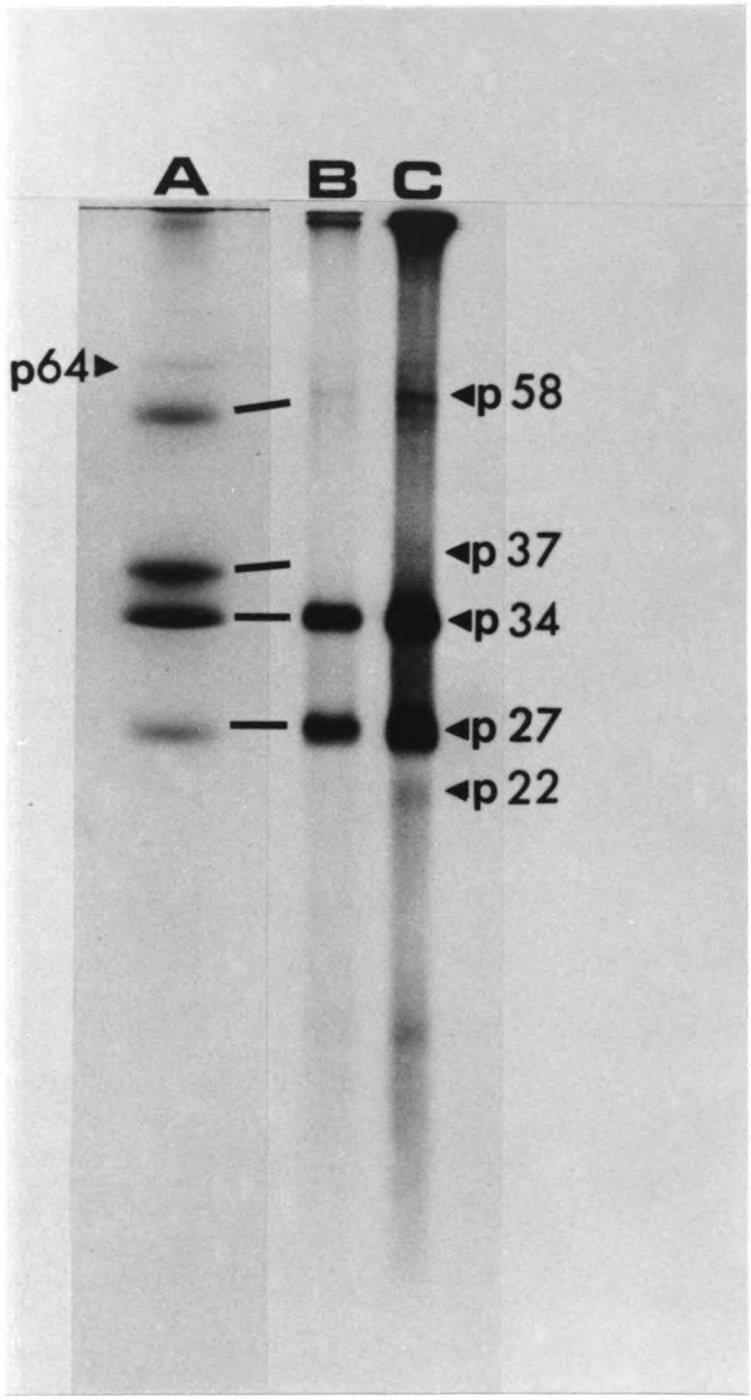
Purified preparations of infectious B-TMEV(WW) were subjected to SDS-PAGE. SDS-PAGE revealed five polypeptides in Coomasie blue-stained gels, the major ones with apparent molecular weight of 58, 37, 34 and 27,000 daltons (Fig. 13, lane A), which were designated P58, P37, P34 and P27 respectively; a minor 64,000 dalton polypeptide was also observed (Fig. 13, lane A). To enhance detection of polypeptides present in low concentrations, purified infectious B-TMEV(WW) was radiolabeled *in vitro* with ^{125}I and subjected to SDS-PAGE. Autoradiography of ^{125}I-labeled virus revealed the same four major polypeptides seen in Coomasie blue-stained gels, plus an additional minor one with an apparent molecular weight of 22,000 daltons, which was designated P22 (Fig. 13, lanes B, C). At excessively long exposures of autoradiograms, a 6,000 dalton polypeptide was detected as well (Fig. 14). No differences were noted in the relative electrophoretic mobilities of the polypeptides detected between ^{125}I-labeled and non-labeled preparations of viral polypeptides or co-electrophoresed molecular weight standards (data not shown). Preparations of B-TMEV(WW) simultaneously iodinated with equal millimolar quantities of nonisotopic iodine were found to retain sufficient infectivity to induce clinical disease when inoculated IC into suckling or weanling mice. Discontinuous SDS-PAGE of Echovirus-12 revealed four major polypeptides with apparent molecular weights of 40, 30, 22, and 10,000 daltons which closely approximate the published reports for VP1, VP2, VP3,

Legend to Figure 13

DISCONTINUOUS SODIUM DODECYL SULFATE-POLYACRYLAMIDE GEL ELECTROPHORESIS OF PURIFIED BRAIN-DERIVED THEILER'S VIRUS (WW STRAIN).

Whole infectious virus was purified by sucrose and CsCl gradient centrifugation following fluorocarbon extraction from infected mouse brain. Virus was subjected to electrophoresis as described in Materials and Methods. The polypeptides were detected by staining with Coomassie Blue (lane A) or autoradiography (lanes B,C) of virus labeled with ^{125}I prior to disruption. Numbers indicate the apparent mol wts (x 10^3 daltons) of the viral polypeptide determined by comparison to co-electrophoresed internal marker proteins. Lane C was loaded with 10X more cpm than lane B and is overexposed to enhance detection of the 58,000 dalton polypeptide. The figure is a composite; lane A is from another electrophoretic run than lanes B and C.

FIGURE 13



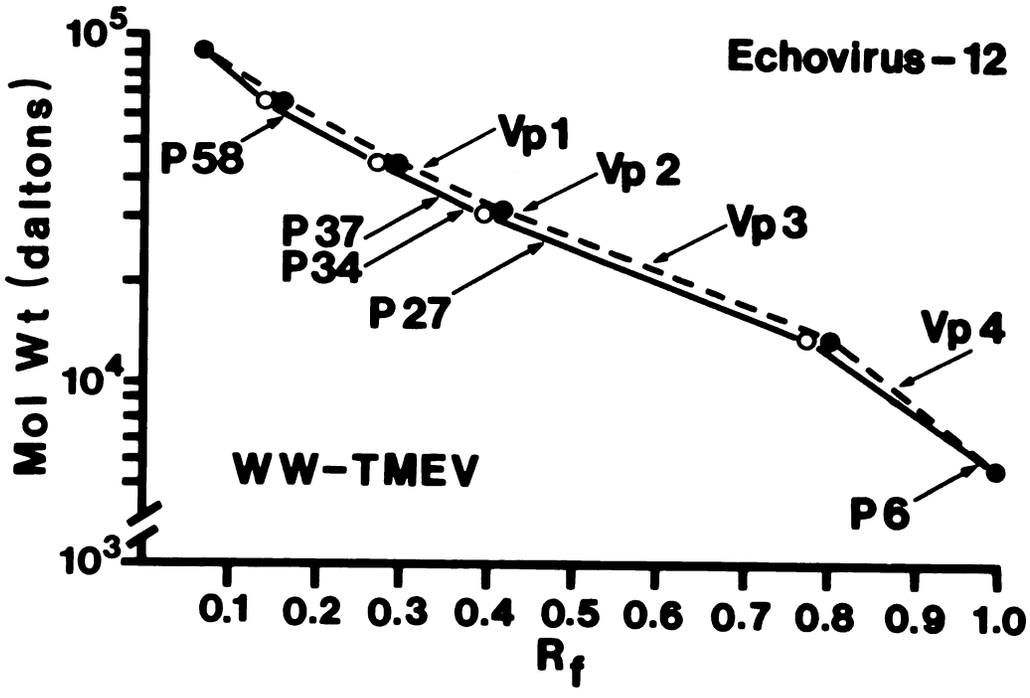


Figure 14

DETERMINATION OF THE MOLECULAR WEIGHTS (MOL WT) OF THEILER'S VIRUS (WW STRAIN) AND ECHOVIRUS-12 POLYPEPTIDES.

Purified virus preparations of Theiler's virus (WW strain) and Echovirus-12 were subjected to electrophoresis with polypeptides of known mol wts (phosphorylase-a, 95,000; bovine serum albumin, 68,000; ovalbumin, 45,000; carbonic anhydrase, 32,000; cytochrome-c, 13,500; insulin, 5750). R_f values of all polypeptides for each run were derived relative to insulin which was arbitrarily defined to have an R_f of 1.0. The position of the major Theiler's virus (WW strain) polypeptides (P58, P37, P34, P27, P6) are indicated by the arrows below the mol wt standard curve for those runs (O———O); Echovirus-12 polypeptides (VP1, VP2, VP3, VP4) are indicated by the arrows above the mol wt standard curves for those runs (●– – – –●).

and VP4 of Echovirus-12 (155) (Fig. 14).

Interestingly, P37 appeared as a major constituent in stained gels (Fig. 13, lane A) but was a minor band in ^{125}I-labeled whole virus preparations (Fig. 13, lanes B, C). These results suggested the possibility that the during the strong oxidation-radiolabeling reaction of whole B-TMEV(WW), the label was attaching preferentially to P37 and P27. To ensure a more complete labeling reaction, purified virus was first disrupted in 5% SDS, followed by iodination and electrophoresis (Fig. 15). SDS disruption of virus prior to iodination provided for a substantial increase in the labeling of P37 of disrupted virions compared to the labeling of P37 in whole virions (Fig. 15).

These results indicated that the major capsid proteins were P37 (VP1), P34 (VP2), P27 (VP3) and P6 (VP4), and that P34 (VP2) and P27 (VP3) were preferentially labeled in whole virions. Less intense iodinations resulted in both P34 (VP2) and P27 (VP3) simultaneously labeling to relatively equal degrees (data not shown).

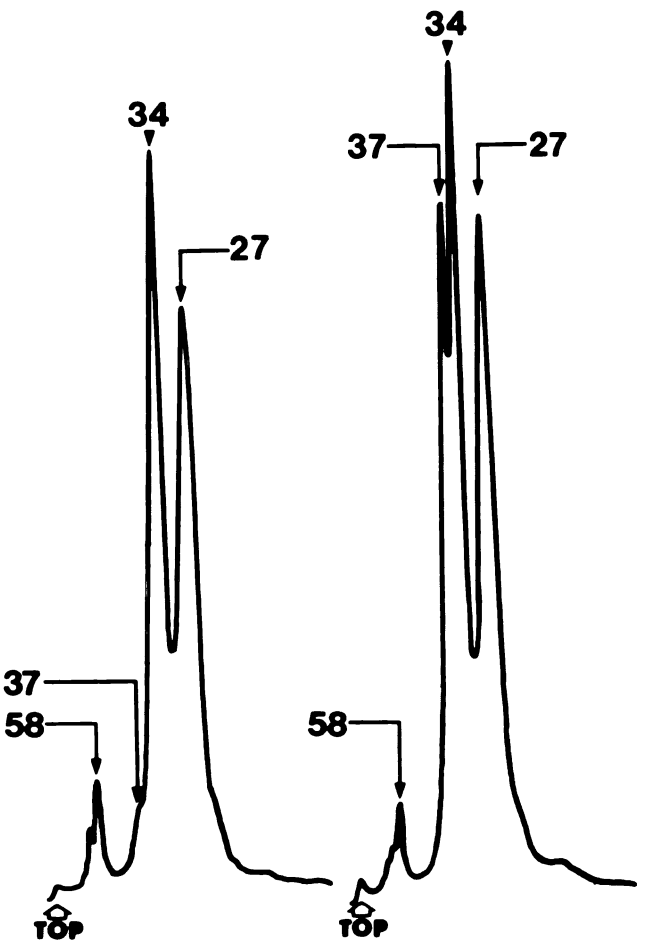
VII. Electrophoresis of B-TMEV(WW) Polypeptides Under Reducing and Non-Reducing Conditions. The relationship of P58 to the capsid polypeptides was investigated. A preparation of purified B-TMEV(WW) was divided into two aliquots; one aliquot was iodinated as whole virus and the second was disrupted in 5% SDS followed by iodination. These preparations were then subjected to co-electrophoresis under reducing and non-reducing conditions.

Legend to Figure 15

DESITOMETRIC SCAN OF AUTORADIOGRAMS OF THEILER'S VIRUS (WW STRAIN) POLYPEPTIDES LABELED BEFORE AND AFTER CAPSID DISRUPTION.

One aliquot of purified, brain-derived whole Theiler's virus (WW strain) was radiolabeled as described in Materials and Methods (left). The other aliquot was disrupted in 5% sodium dodecyl sulfate prior to radiolabeling (right). Unbound ^{125}I was removed from both samples by gel filtration and the samples co-electrophoresed. P6 in not visible in these densitometric tracings because the long exposures required to detect it resulted in overexposure of P58, P37, P34 and P27. Numbers refer to the mol wts of the polypeptides (x 10^3 daltons).

FIGURE 15



When virus was disrupted in SDS prior to iodination, and reduced in 2-mercaptoethanol (2-ME), little if any P58 was detectable (Fig. 16, lanes E,F). P34 (VP2) and P27 (VP3) appeared to be slightly enhanced relative to P37 (VP1). In contrast, P58 was detectable in virus preparations electrophoresed under non-reducing conditions (Fig. 16, lane D). P64 was not detectable under any condition in which virus was disrupted prior to radiolabeling.

When identical experiments were performed using whole radiolabeled virus, similar results were obtained (Fig. 16, lanes A-C). P58 was present in such preponderance under non-reducing conditions that had overloaded this region of the gel (Fig. 16, lane C). The relative quantitiy of P58 decreased as the percent of 2-ME increased (compare lanes A and B with C, Fig. 16). P40 was detected in these gels and was presumably the VP0 of B-TMEV(WW) as it was only occasionally seen (see Introduction, Table 6). P22 was only present in the lanes of whole radiolabeled virus (compare lanes A-C with D-F, Fig. 16).

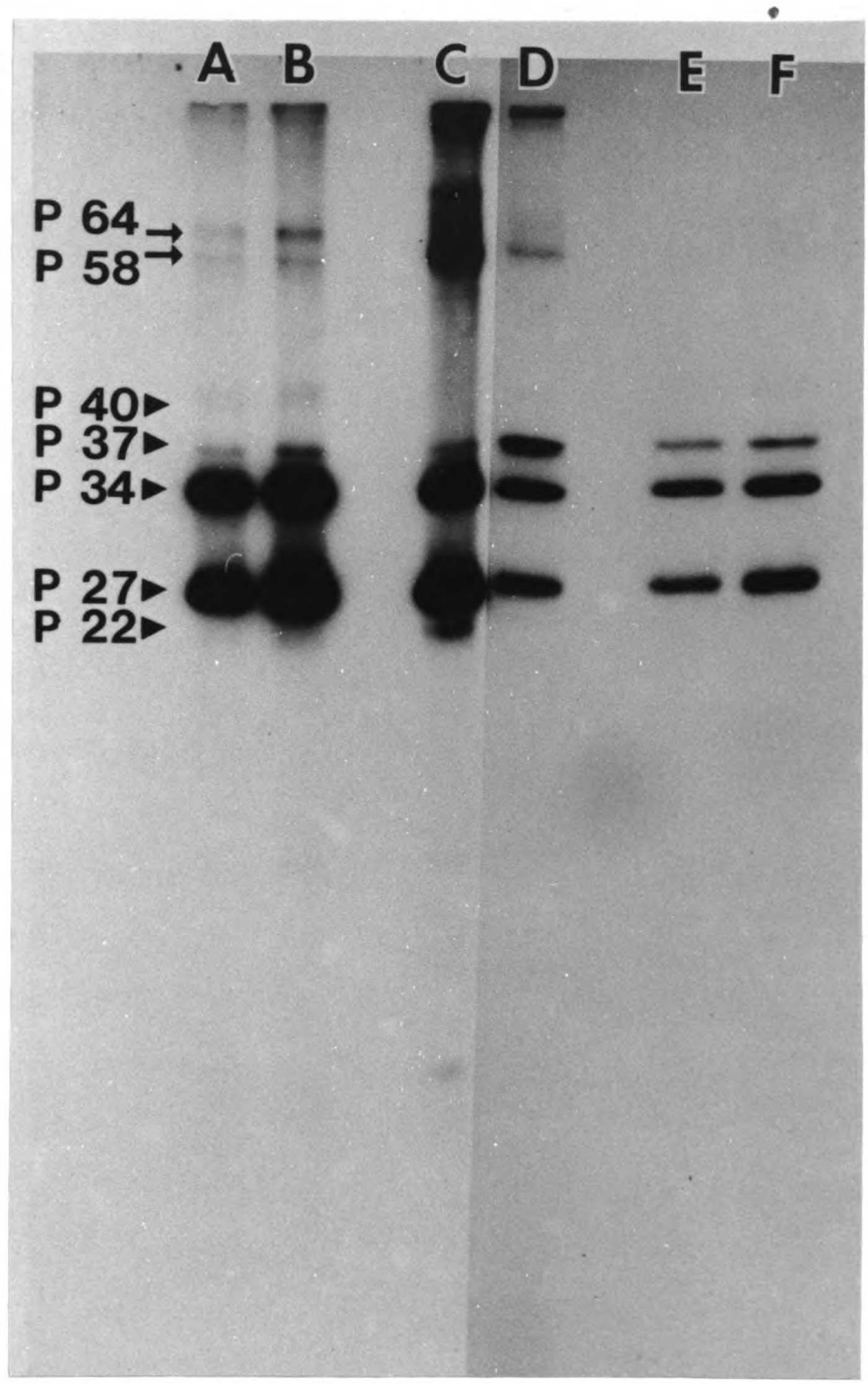
VIII. P34 (VP2) and P27 (VP3) are Derived From B-TMEV(WW) P58. P58 appeared to be a major constituent in gels of both whole and disrupted, radiolabeled virus preparations. Since the results obtained in the above experiments indicated that P58 seemed to decrease in quantity as the strength of the reducing conditions increased, a means to isolate P58 and characterize its reduced products was developed. The experimental approach was to electrophorese

Legend to Figure 16

DISCONTINUOUS SODIUM DODECYL SULFATE-POLYACRYLAMIDE GEL ELECTROPHORESIS OF BRAIN-DERIVED THEILER'S VIRUS (WW STRAIN) LABELED WITH ^{125}I BEFORE (WHOLE VIRUS) OR AFTER (DISRUPTED VIRUS) CAPSID DISRUPTION, AND ELECTROPHORESED UNDER REDUCING AND NON-REDUCING CONDITIONS

Whole virus (lanes A,B, and C) was prepared for electrophoresis in the presence of 4% 2-ME (lane A), 2% 2-ME (lane B) and without 2-ME (lane C). Disrupted virus (lanes B,E, and F) was prepared by heating whole virus in 5% SDS for 2 min prior to radiolabeling. Disrupted virus was then prepared for electrophoresis in the presence of 4% 2-ME (lane F), 2% 2-ME (lane E) and without 2-ME (lane D). All samples contained equivalent cpm and were co-electrophoresed. The figure is a composite of two different exposures of the same autoradiogram. Numbers refer to the mol wts of the polypeptides (x 10^3 daltons).

FIGURE 16



whole radiolabeled virus under non-reducing conditions, locate P58, remove it from the gel and subject it to re-electrophoresis under reducing conditions. The autoradiogram of one such experiment is shown in Figure 17. These results demonstrated that P34 (VP2) and P27 (VP3) were derived from P58 by reduction. It was of interest that not all of the P58 was reducible under these conditions, which was an agreement with the results in Figure 15. Prolonged exposures of these autoradiograms failed to show any other reduction products.

To confirm that P34 (VP2) and P27 (VP3) were derived from the large apparent precursor, P58, limited proteolysis in gel was performed on polypeptides P58, P34 and P27, using a modification of the methods of Cleveland, et. al. (47). Polypeptides were first separated from each other by SDS-PAGE under non-reducing conditions, located, and re-electrophoresed under reducing conditions in the presence of Staphylococcus aureus protease V8. Proteolytic digestions were carried out at 22^{o}C in the gel, at enzyme:substrate excess. One such experiment is illustrated in Figure 18. The large peptide fragments of P27 (b and d) and P34 (c) corresponded to the larger peptide fragments of P58 (b, c, and d), and were unique to P27 and P34; other smaller fragments (e-i) were common to P34, P27 and P58. These results demonstrated that P58 was the progenitor of P34 and P27.

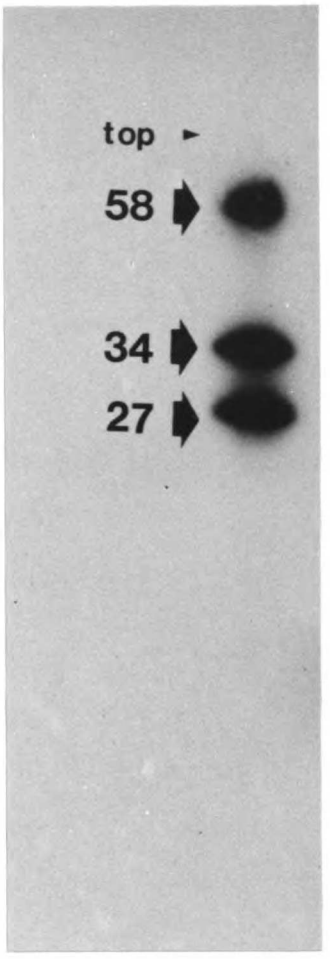


Figure 17

RE-ELECTROPHORESIS UNDER REDUCING CONDITIONS OF BRAIN-DERIVED VIRUS (WW STRAIN) P58 ISOLATED FROM A GEL ELECTROPHORESED UNDER NON-REDUCING CONDITIONS.

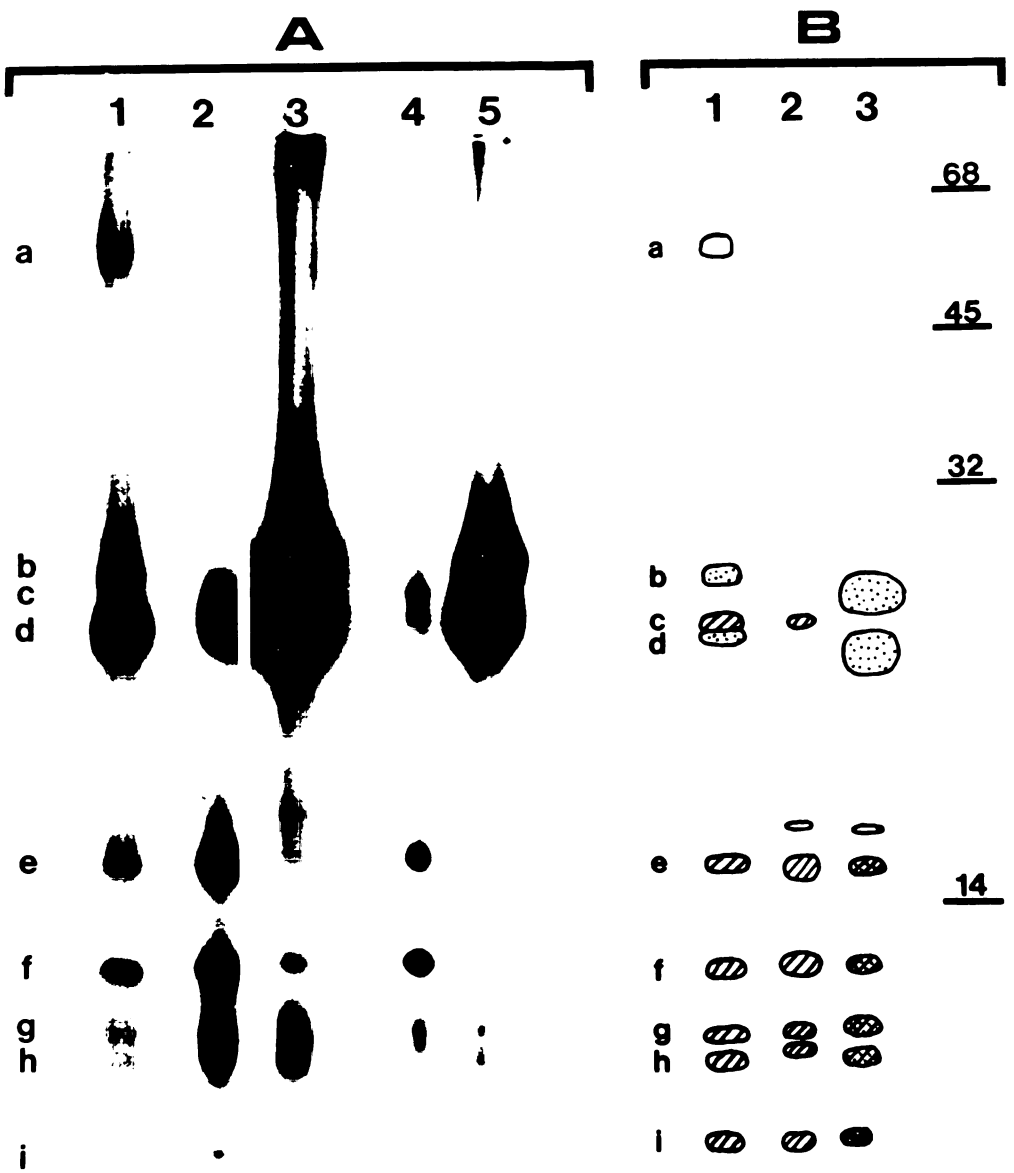
P58 was located in a gel of whole radiolabeled brain-derived Theiler's virus (WW strain) run under non-reducing conditions and prepared for re-electrophoresis as described in Materials and Methods. Numbers refer to the mol wts of the polypeptides (x 10^3 daltons).

Legend to Figure 18

PEPTIDE MAPPING OF BRAIN-DERIVED THEILER'S VIRUS (WW STRAIN) (B-TMEV(WW)) POLYPEPTIDES P58, P34 AND P27 BY LIMITED PROTEOLYSIS IN GEL.

Polypeptides P58, P34 and P27 were removed from a gel of whole B-TMEV(WW) electrophoresed under non-reducing conditions as described in Materials and Methods and subjected to limited proteolysis in gel according to the methods of Cleveland, et. al. (47). Protease V8 was the enzyme used to generate the fragments (a-i). (A) Autoradiograms of fragments of P58 (A-1), P34 (A-2,-4), P27 (A-3,-5). Lanes A-2,-3 are overexposed to show e-h; lane A-5 is underexposed to discern P27 fragments b,d from P34 fragment c (A-4,-2). (B) Tracings of the lanes shown in (A). Fragments in common between P58 (B-1) and P27 (B-2) are indicated by (///); those common to P58 and P34 (B-3) are indicated by dots. Fragments unique to any polypeptide are without hatching or dots. Fragment "a" probably represents undigested P58. Numbers and lines indicate mol wt and position of marker proteins. (A) and (B) are composites of different lanes and autoradiograms of the same gel. Different apparent specific activities of fragments is probably due to unequal radiolabeling of P58, P34 and P27 in whole virus.

FIGURE 18



IX. Effects of Trypsin on B-TMEV(WW) Polypeptides.

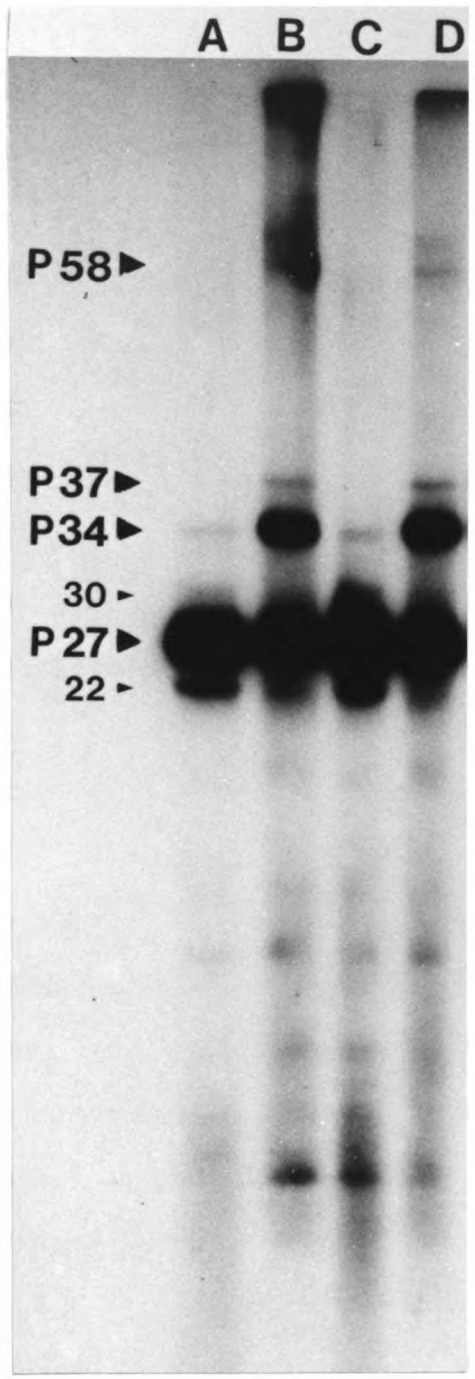
The radiolabeling characteristics of whole virus suggested that P34 (VP2) and P27 (VP3) were oriented on the surface of the virus and that P37 (VP1) was located more internally. Consequently, the trypsin sensitivity of B-TMEV(WW) polypeptides was investigated. Preparations of whole and SDS disrupted ^{125}I-labeled TMEV were made and one aliquot was treated for 20 minutes at 37^{o}C with 100 ug of trypsin in 0.02 M Tris-HCl buffer pH 7.2. Control aliquots containing equivalent cpm were incubated with an equivalent amount of buffer without trypsin. Preliminary experiments had indicated that the maximum effect of trypsin treatment was achieved after 20 minutes of incubation making longer digestion time unnecessary. Following digestion, the samples were then subjected to electrophoresis under non-reducing and reducing conditions (Fig. 19). When the virus was incubated with trypsin, several cleavages were noted to occur (Fig. 19, lanes A, C). Under reducing and non-reducing conditions, trypsin had the effect of completely digesting P58, P37 (VP1) and nearly all of P34 (VP2). P22, a minor component observed in whole virus preparations was markedly enhanced by trypsin treatment. Presumably P22 was a specific cleavage product of either P58, P37 (VP1) or P34 (VP2) and appeared in some autoradiograms due to opportunistic proteases with trypsin-like activity in preparations of purified whole radiolabeled virus. A new poly-

Legend to Figure 19

EFFECT OF TRYPSIN ON ^{125}I-LABELED BRAIN-DERIVED THEILER'S VIRUS (WW STRAIN).

Purified whole brain-derived Theiler's virus (WW strain) was radiolabeled with ^{125}I and incubated with or without trypsin at 37^{o}C for 20 min immediately prior to co-electrophoresis under reducing (R) and non-reducing (NR) conditions. Lane A: R, + trypsin; lane B: NR, -trypsin; lane C: NR, + trypsin; lane D: R, -trypsin. All lanes were loaded with equal cpm. Numbers refer to the mol wts of the polypeptides (x 10^3 daltons).

FIGURE 19



peptide, P30, was also observed following trypsin treatment. Presumably it too was derived from one of the larger polypeptides.

There were numerous other small polypeptides in the lanes containing whole B-TMEV(WW) (Fig. 19, lanes A, C); these probably represented specific cleavage products of the major polypeptides, but interpretation of them was not possible with the techniques employed. Trypsin treatment of SDS disrupted virus preparations was unsuccessful due to the high percent of SDS present in these samples.

X. Immunoreactivity of B-TMEV(WW) P58. The results obtained indicated that P58 of B-TMEV (WW) was a surface oriented polypeptide as assessed by its reactivity during the iodination reaction and by its sensitivity to trypsin, and was a precursor of capsid proteins P34 (VP2) and P27 (VP3). The fact that P58 could be reduced into two of the capsid proteins, P34 (VP2) and P27 (VP3), and shared peptide fragments with P34 (VP2) and P27 (VP3) strongly argued that P58 was not a contaminant. Immunoprecipitation experiments were performed, however, to determine whether P58 was indeed a viral specific polypeptide and not a tenacious host contaminant which could coincidentally be reduced into two polypeptides which comigrated with P34 (VP2) and P27 (VP3).

These experiments were performed by reacting each viral polypeptide isolated from polyacrylamide gels run under non-reducing conditions with specific Theiler's virus (WW strain) antiserum. The immune complexes formed were immunopre-

cipitated by addition of a suspension of Staphylococcus aureus, which preferentially binds immune complexes. The immune complexes bound to S. aureus were washed several times until stable amounts of radioactivitiy were obtained in the pellet and supernatant fractions. The specific reactivity of the viral polypeptides was determined by comparing the percent of the viral protein bound in the presence of specific antiserum to the percent bound in the presence of pre-immune rabbit serum. The results of these experiments are illustrated in Table 8. These results indicated that P58, P34, P27 and P6 were immunoreactive with specific WW strain antiserum to a greater extent than whole virus, treated in an identical fashion. The specific reactive index of whole virus (1.64) represents a 58% increase in bound radioactivity over the nonspecific reactive index. This value was considered the minimum positive increase in percent reactivity and all viral polypeptides were considered to positively specifically react wtih antibody if their percent increases of specific reactivity to nonspecific reactivity were greater than 58%.

The most likely explanation for the variability in percent increases of specific to nonspecific reactive indices probably relates to the unequal denaturation, differences in epitope presentation of the solubilized, extracted viral polypeptides vis a vis native or intact whole virus, as well as differences in specific activities (cpm:ug

Table 8: Immunoprecipitation of Whole ^{125}I-Labeled Brain-Derived Theiler's Virus (WW Strain) and Viral Polypeptides Isolated From Polyacrylamide Gels

	$\bar{X}\% \pm 1$ SD[a] Bound CPM	NSRI[b]	SRI[c]
Whole Virus			
Virus without Serum	12.6 ± 2.6		
Virus + NRS[d]	13.0 ± 2.3	1.04	
Virus + Antibody[e]	20.5 ± 1.0		1.64 (58)[f]
Viral Polypeptides			
Proteins - Serum			
P 58	9.4 ± 2.6		
P 34	5.6 ± 0.2		
P 27	7.0 ± 2.0		
P 6	3.3 ± 0.4		
Proteins + NRS			
P 58	8.2 ± 0.6	0.87	
P 34	4.1 ± 0.6	0.73	
P 27	6.2 ± 2.3	0.88	
P 6	3.0 ± 0.1	0.91	
Proteins + Antibody			
P 58	13.6 ± 0.4		1.65 (90)
P 34	15.1 ± 1.2		3.65 (400)
P 27	8.7 ± 0.7		1.40 (59)
P 6	5.9 ± 0.9		1.96 (115)

[a]SD: Standard Deviation

[b]NSRI: Nonspecific Reactive Index (see Materials and Methods)

[c]SRI: Specific Reactive Index (see Materials and Methods)

[d]NRS: Normal Rabbit Serum from a rabbit inoculated with normal mouse brain homogenate (see Materials and Methods)

[e]Antibody: Theiler's Virus (WW Strain) specific rabbit antiserum

[f]Numbers in parantheses are percent increases of SRI over NSRI for each sample

protein) of the extracted polypeptides themselves. When identical experiments were performed using heterotypic antisera (anti-GDVII strain) essentially identical results were obtained.

The immunoprecipitation experiments described above were performed using solubilized proteins from polyacrylamide gels. This experimental approach was chosen because, if the postulate that P58 was a tenacious host contaminant was correct, direct immunoprecipitation of whole virus and elucidation of which polypeptides were immunoprecipitable by SDS-PAGE analysis, would have potentially resulted in immunoprecipitation of P58, through a "carrier effect" and given a false positive result. However, because the above experiments indicated that P58 was specifically immunoreactive, and therefore a viral specific polypeptide, direct immunoprecipitation experiments of whole virus were performed in a manner similar to the experiments described above. These experiments confirmed the above studies.

XI. Comparison of the Polypeptides of Brain-Derived and Tissue Culture Adapted Theiler's Virus (WW Strain). Purified preparations of B-TMEV(WW) and TC-TMEV(WW) were radiolabeled under identical conditions with ^{125}I before and after capsid disruption and submitted to SDS-PAGE under reducing and non-reducing conditions. The studies outlined above indicated that whole B-TMEV(WW) P58 overloaded the top region of the gel when run under non-reducing conditions

(see Fig. 16). Therefore, to facilitate comparison of TC- and B-TMEV(WW) polypeptides in this region of the gel, B-TMEV(WW) was prepared in reducing sample buffer containing 2% 2-ME which decreased this problem (compare lanes B and C, Fig. 16) and the other samples of TC-TMEV(WW) were prepared in 4% 2-ME. Figure 20 compares the polypeptides of B- and TC-TMEV(WW). Both viruses contained relatively equal quantities of picornaviral capsid polypeptides VP1 (P37), VP2 (P34), and VP3 (P27) as assessed in viral preparations radiolabeled after capsid disruption (Fig. 20, lanes B, C). The fourth picornaviral capsid polypeptide, VP4 (P6) could only be detected in autoradiograms after long exposures of gels. Traces of VP0 (P40), a procapsid precursor of VP2 and VP4 (279) were occasionally seen in both B- and TC-TMEV(WW) (Fig. 20). Both viruses contained P64, but interestingly, TC-TMEV(WW) lacked P58 present in B-TMEV(WW) (compare lanes A and B with C and D, Fig. 20). A 44,000 dalton mol. wt. polypeptide was occasionally observed in whole TC-TMEV(WW) run under reducing conditions (Fig. 20, lane C) but the significance of this polypeptide was not investigated because it was observed rather rarely. More intense iodination conditions and prolonged exposures of autoradiograms failed to demonstrate P58 in TC-TMEV(WW) (data not shown).

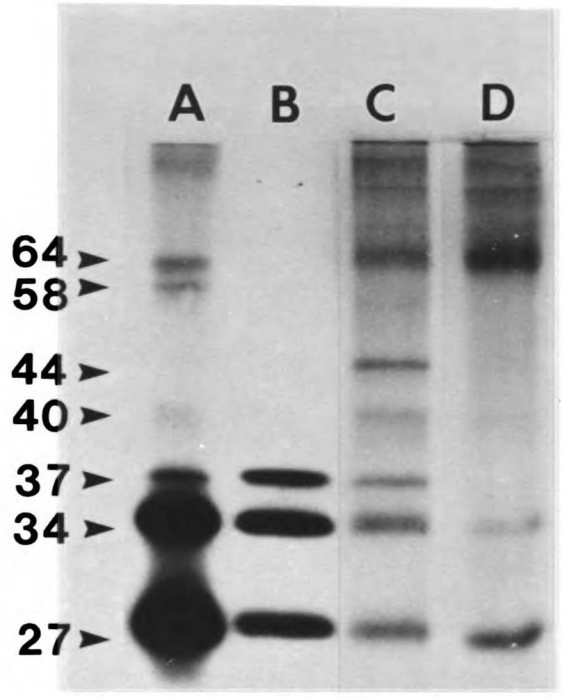
With the exception of the absence of P58 in TC-TMEV(WW), comparison of electrophoretic profiles of viruses labeled before and after capsid disruption did not reveal any major differences in surface oriented tyrosine residues in capsid

Legend to Figure 20

COMPARISON OF THE POLYPEPTIDES OF BRAIN-DERIVED (B-) AND TISSUE CULTURE ADAPTED (TC-) THEILER'S VIRUS (WW STRAIN) (TMEV(WW)).

Purified preparations of B-TMEV (WW) (lanes A and B) and TC-TMEV(WW) (lanes C and D) were radiolabeled before (lanes A and C) and after (lanes B and D) capsid disruption. Whole B-TMEV(WW) was prepared for electrophoresis in sample buffer containing 2% 2-ME (lane A) to allow for detection of P58 and P64; all other samples were prepared in sample buffer containing 4% 2-ME. The figure is a composite of two exposures of the same autoradiogram. Numbers refer to the mol wts of viral polypeptides (x 10^3).

FIGURE 20



polypeptides between TC- and B-TMEV(WW). In both viruses, VP2 and VP3 appeared to have more tyrosine residues available to react during the iodination reactions of whole virions than VP1 (Compare lanes B and C with A and D, Fig. 20). SDS disruption of virions prior to radiolabeling allowed for more complete labeling of VP1 (P37) in both B- and TC-TMEV(WW) (compare lanes A and B with C and D, Fig. 20). TC-TMEV(WW) P64 and B-TMEV(WW) P58 and P64 had tyrosine residues accessible during the radiolabeling reaction of whole virions as well in agreement with Figure 16. These results indicated that the major biochemical difference between B- and TC-TMEV(WW) was the presence of P58 in preparations of B-TMEV(WW).

XII. Analysis of Tissue Culture Adapted Theiler's Virus (WW Strain) P64. Comparison of whole virus preparations run under non-reducing and reducing conditions indicated that B-TMEV (WW) P58 was reducible, whereas P64 of TC-TMEV(WW) was not (Compare lanes A, B and C, Fig. 16 and lane A with lanes C and D, Fig. 20). Because B-TMEV(WW) P58 was found to be reducible into VP2 (P34) and VP3 (P27) when isolated from a gel and subjected to re-electrophoresis under reducing conditions (see Fig. 17), TC-TMEV(WW) P64 was subjected to the same analysis.

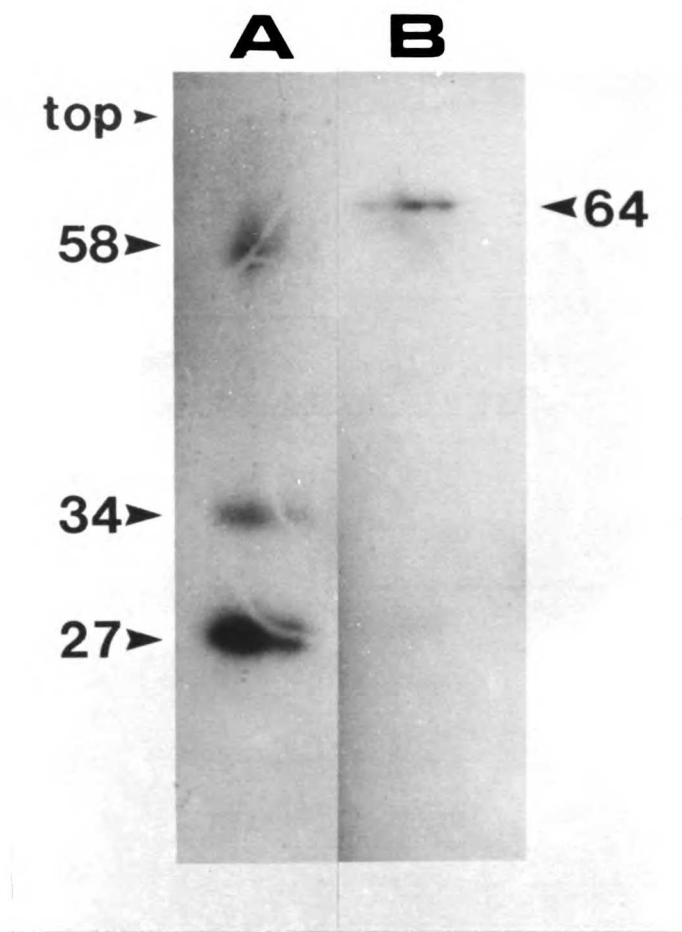
Figure 21 illustates that P64 of TC-TMEV(WW) was not reduced into VP2 (P34) and VP3 (P27) to the same extent as B-TMEV(WW) P58, and that TC-TMEV(WW) polypeptide P44 was not a reduction product of P64. These results confirmed the

Legend to Figure 21

RE-ELECTROPHORESIS OF BRAIN-DERIVED THEILER'S VIRUS (WW STRAIN) (B-TMEV(WW)) P58 AND TISSUE CULTURE ADAPTED THEILER'S VIRUS (WW STRAIN) (TC-TMEV(WW)) P64 UNDER REDUCING CONDITIONS.

Preparations of whole ^{125}I labeled B- and TC-TMEV(WW) were electrophoresed under non-reducing conditions. Polypeptides were isolated from the non-reducing gel and prepared for re-electrophoresis under reducing conditions in sample buffer containing 2% 2-ME. Lane A, re-electrophoresis of B-TMEV(WW) P58; lane B, re-electrophoresis of TC-TMEV(WW) P64. Numbers refer to the mol wts of polypeptides (x 10^3 daltons). The figure is a composite of two lanes of the same autoradiogram.

FIGURE 21



observation that TC-TMEV(WW) P64 was not reducible (compare lanes C and D, Fig. 20), whereas B-TMEV(WW) P58 was reducible.

XIII. Assessment of the Biological Significance of P58. The above results indicated that the major biochemical difference between B- and TC-TMEV(WW) was that B-TMEV(WW) contained a percent of virions bearing a surface oriented, reducible precursor of capsid proteins VP2 (P34) and VP3 (P27). This evidence indicated that the presence of P58 in a viral inoculum was related to the ability of that inoculum to induce acute polioencephalomyelitis (see Histopathology and Clinical Course above).

Attempts were made to directly determine if P58 correlated with the encephalitogenic potential of B-TMEV(WW). The use of mice for assessment of the effect of 2-ME treatment on B-TMEV(WW) was dictated by two factors. First, the experiments were designed to assess the effect of chemical treatment on encephalitogenicity, which can only be measured in vivo. Second, the effect of 2-ME treatment on B-TMEV(WW) could not be assessed in tissue culture because of the inability of brain-derived virus to cause direct CPE. Mice were IC inoculated with B-TMEV(WW) which had been preincubated with 2-ME to reduce P58. The ratio to 2-ME:virus was proportional to the ratio used in electrophoresis under reducing conditions (see legend to Fig. 16, lane B). One group of control mice received equivalent amounts of virus prepared in an identical fashion but without 2-ME, and

another control group received identical concentrations of 2-ME only. No toxicity was observed in control mice which received between 2%-4% 2-ME.

The results of one of several experiments are shown in Figure 22 and Table 9. The group which received 2-ME treated virus exhibited a delay in the onset of morbidity and mortality compared to the control group which received untreated virus. Furthermore, 2-ME treatment of B-TMEV(WW) resulted in a significant difference in the number of moribund and dead animals at 10 days post-inoculation ($x^2=4.722$, $p < 0.05$) and at 11 days post-inoculation ($x^2=3.820$, $p < 0.1$) compared to the group which received untreated virus.

When identical experiments were performed on TC-TMEV(WW), and the effects of 2-ME treatment assessed in vitro, it was found that 2-ME treatment did not reduce viral titers or alter the evolution of CPE during the course of infection of the monolayers. In vivo titration of 2-ME treated TC-TMEV (WW) was not performed because the low frequency and subtlety of clinical signs in TC-TMEV(WW) infected mice would have made an endpoint of the titration impossible to detect. These results indicated that P58 of B-TMEV(WW) was related to the ability of the virus to induce acute polioencephalomyelitis, and that 2-ME treatment of P64 bearing virions (TC-TMEV(WW)) did not result in a loss of infectivity.

Legend to Figure 22

EFFECT OF 2-MERCAPTOETHANOL (2-ME) TREATMENT OF PURIFIED BRAIN-DERIVED THEILER'S VIRUS (WW STRAIN) (B-TMEV(WW)) ON APPEARANCE OF NEUROLOGIC DISEASE FOLLOWING INTRACRANIAL INOCULATION OF WEANLING MICE.

Two groups of mice (N = 20) were inoculated with a preparation of B-TMEV(WW) which had been incubated with 2-ME prior to inoculation (□——□), and another group (N = 20) was inoculated with virus which was incubated in buffer without 2-ME (●——●). Both preparations contained $10^{5.4}$ LD_{50} (suckling mouse doses) of infectious virus before 2-ME treatment. Mice were observed for appearance of hind limb paralysis at intervals after infection. 2-ME treatment provided for a significant decrease in the number of sick and dead animals at 10 ($x^2 = 4.722$, $P < 0.05$, with Yate's correction) and 11 ($x^2 = 3.820$, $P < 0.1$, with Yate's correction) days post inoculation.

FIGURE 22

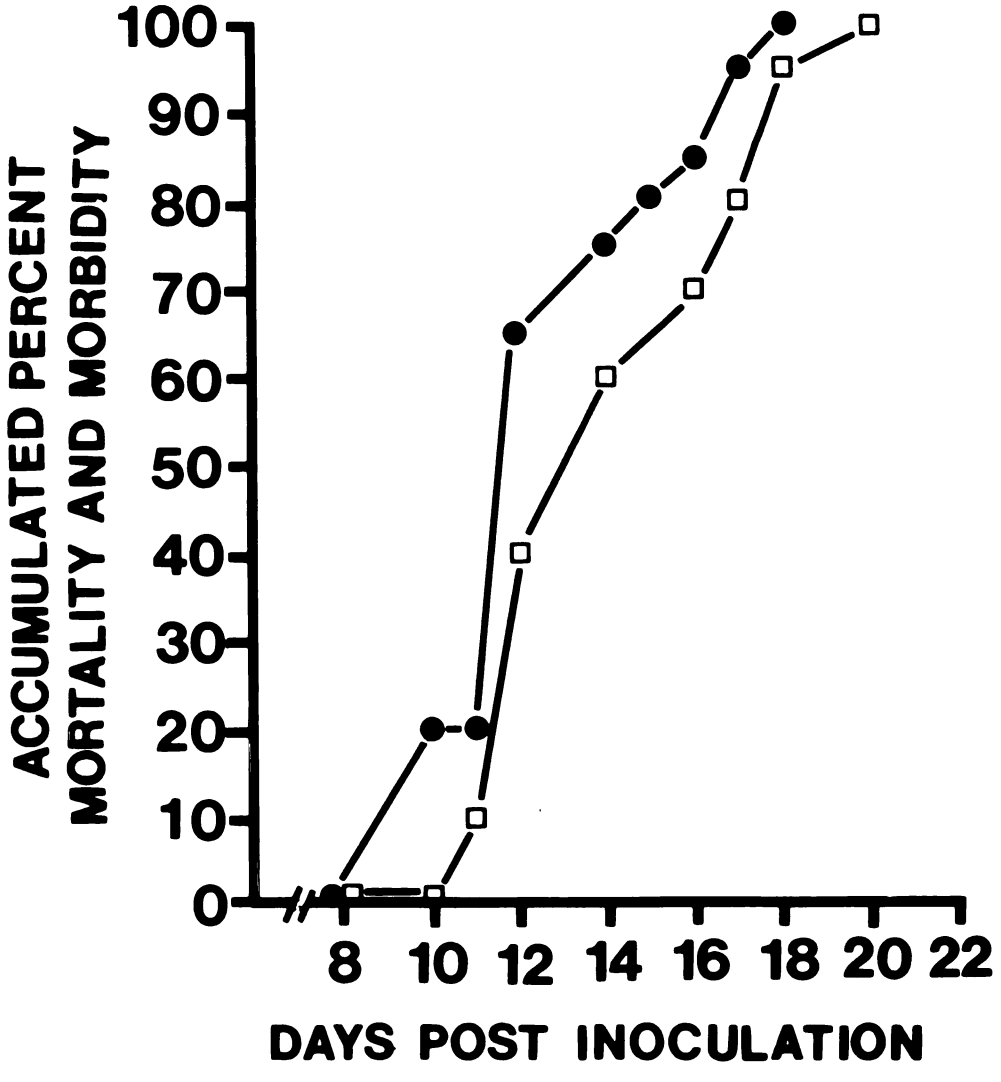

ACCUMULATED PERCENT
MORTALITY AND MORBIDITY
100
90
80
70
60
50
40
30
20
10
0
8
10
12
14
16
18
20
22
DAYS POST INOCULATION

Table 9: Effects of 2-Mercaptoethanol (2-ME) Treatment of Brain-Derived Theiler's Virus (WW Strain) on Pathogenesis In Vivo

OBSERVATION WITHIN GROUP	UNTREATED VIRUS RECIPIENTS	2-ME TREATED VIRUS RECIPIENTS
1st Day of Bilateral Paralysis	10	12
1st Day of 100% Morbidity	18	20
1st Day of Death	11	14
Day of 100% Mortality	11	29

DISCUSSION

One of the purposes of these studies was to characterize the biophysical and biochemical properties of the WW strain of Theiler's virus, and compare these properties with those of more well characterized picornaviruses. Infectious picornavirions are nonenveloped icosahedral particles with a diameter of about 30 nm, have an average sedimentation coefficient of 156 ($S_{20}\omega$), characteristically band in CsCl at a bouyant density of about 1.34-1.35 gm/cm^3, and contain an RNA genome which has a sedimentation coefficient of 35S (see Introduction, Table 6). The capsid polypeptides of mature picornaviruses consist of four species with molecular weights of 28-36,000 (VP1), 26-32,000 (VP2), 21-30,000 (VP3) and 5.5-14,000 daltons (VP4) (see Introduction, Table 6). Most picornaviruses probably contain equal molar quantities of the capsid proteins; however, their orientation in the virus particle is still not completely understood. Occasionally, traces of a fifth polypeptide, VP0, with a molecular weight of about 40,000 daltons, are also observed (see Introduction, Table 6).

The studies reported here indicated that Theiler's virus (WW strain) fulfill these defining criteria for inclusion in the Picornaviridae. Both tissue culture (TC-) and brain-derived (B-) Theiler's virus (WW strain) (TMEV(WW)) were found to be 26 nm particles which had a sedimentation

coefficient of 156 ($S_{20}\omega$) in rate-zonal sucrose gradients. Both B- and TC-TMEV(WW) banded in CsCl gradients at a bouyant density of 1.34-1.35 gm/cm^3. The conventional nomenclature (VP0-VP4) to designate picornaviral proteins may be premature for TMEV(WW), as the polyprotein cleavage-assembly process (see Introduction, Figure 1) of TMEV has not yet been reported. However, two lines of experimental evidence strongly suggest that B- and TC-TMEV(WW) P37, P34 and P27 are three of the four structural polypeptides of the virus, VP1, VP2 and VP3.

First, in experiments where viral polypeptides were detected in polyacrylamide gels by staining, three major bands were detected, P37, P34 and P27 (in addition to P58 and P64) (Fig. 13, lane A). Second, when virions were treated with SDS, under conditions that insure complete disruption of picornaviral capsids (187), radiolabeled and electrophoresed, three predominant species, P37, P34 and P27, were detected in nearly equal quantities (Fig. 20, lanes B, C; Fig. 15). During performance of these studies, Lipton et al. described the polypeptide composition of several strains of tissue culture adapted Theiler's viruses (176). Although these investigators did not report the sedimentation velocities of the viruses studied, the results reported here are essentially in agreement with their results; the modest differences in the molecular weights of TMEV polypeptides probably reflect slight differences in molecular weight calibration curves and other variations of

SDS-PAGE that differ among laboratories. The smallest structural polypeptide, VP4, has always been difficult to demonstrate in picornaviruses, but was reported by Lipton, et. al. in tissue culture TMEV (176), and was observed in these studies in both B- and TC-TMEV(WW). VP4 may be more difficult to demonstrate, however, when TMEV was radiolabeled in vitro with ^{125}I than when radiolabeled in tissue culture. Taken together, these data indicate that TMEV(WW) does contain the four typical capsid polypeptides seen in all picornaviruses.

Although the genome of TMEV is known to be RNA (89), this report describes the first characterization of its sedimentation behavior in rate-zonal sucrose gradients. These studies have demonstrated that the genome of B-TMEV(WW) consisted of a 35S RNA (Fig. 12). Although RNA extraction was performed using the classic SDS-citric acid method (190, 191) following solubilization of the capsid in SDS and proteinase-K, incomplete removal of the protein from the RNA often resulted. Complete removal of protein was accomplished by further extraction of the RNA in chloroform-phenol (see Materials and Methods). The presence of contaminating protein in preparations of SDS-citric acid extracted RNA could be readily visualized as an opalescent band appearing at the aqueous-chloroform-phenol interface during the phenol extraction step. RNA, free of contaminating protein, was centrifuged through rate-zonal sucrose gradients containing 0.5% SDS. The majority of the RNA sedimented faster than

the rRNA markers centrifuged under identical conditions, and had an estimated sedimentation coefficient of 35S (205).

These procedures have been used to analyse the sedimentation characteristics of many picornaviral genomes (100, 304) and separate intact 35S RNA from sub-genomic length RNA molecules. The faster sedimenting trailing edge of B-TMEV(WW) 35S RNA is probably due to some artificial secondary structure of the RNA caused by partial ionic charge repulsion in the sucrose gradient due to the ionic strength of the buffer system used. When other rate-zonal centrifugations were performed using gradients containing 50% dimethyl sulfoxide (DMSO), to denature the RNA, nearly all of the RNA was degraded into small fragments which had sedimentation coefficients of approximately 16S or less. But, because the rate-zonal sucrose gradient depicted in Figure 12 was performed under conditions which have been used for other picornaviral RNAs, it is probable that the 35S RNA observed was representative of the B-TMEV(WW) genome.

The studies reported here shed light on the orientation of the capsid polypeptides of TMEV(WW). Most picornaviruses appear to have VP1 as the major capsid protein as assessed by reaction of intact virions with iodine (39, 179), N-succinimidyl propionate (343) and acetic anhhydride (179) (see Introduction, "Structure, Antigenicity and Morphogenesis of Picornaviruses"). The biologic importance of the surface orientated VP1 as a determinant of virus-cell interaction is further supported by antibody blocking and

protease susceptibility experiments (43, 182). These reports suggested that VP1 is a major contributor to the surface structure of picornaviruses, and may be chiefly involved in host cell recognition. However, VP1 is not the exclusive surface protein of picornaviruses. Recent evidence obtained with poliovirus treated with the Bolton-Hunter reagent indicated that VP3 also had a surface orientation (343). When both B- and TC-TMEV(WW) were disrupted in SDS, labeled and electrophoresed, nearly uniform labeling of P37 (VP1), P34 (VP2) and P27 (VP3) was achieved, indicating that each protein had nearly equivalent percents of tyrosine residues. This is in agreement with the fact that the calculated percent of tyrosine residues per capsid protein for ME virus, mengovirus and foot-and-mouth disease virus are about the same (279). However, when whole virions were radiolabeled and electrophoresed, the majority of the label was found in P34 (VP2) and P27 (VP3).

These data suggested that either P34 (VP2) and P27 (VP3) had more of a total surface orientation than P37 (VP1), or that the labeled tyrosine residues of P37 (VP1) were more externally located relative to the tyrosine residues of P34 (VP2) and P27 (VP3). The latter interpretation was supported by the trypsin digestion experiments on whole radiolabeled B-TMEV(WW). Trypsin had the effect of cleaving off all labeled residues of P37 (VP1), most residues of P27 (VP3), and left the residues of P27 (VP3) apparently unaffected (Fig. 19). The labeling character-

istics of TC-TMEV(WW) suggested the same orientation of capsid polypeptides as B-TMEV(WW). These experiments indicated that under all radiolabeling conditions used, P34 (VP2) and P27 (VP3) had more tyrosine residues accessible to the radiolabeling reagents than P37(VP1), but that of all labeled residues, those of P37 (VP1) were most externally located and susceptible to complete cleavage by trypsin. More experiments are required to assess whether all of the P37 (VP1) polypeptide is surface oriented.

These studies document one notable exception to the structural similarity between other picornaviruses and TMEV(WW), the presence of high molecular weight polypeptides (P64 and P58) in preparations of highly purified B- and TC-TMEV(WW). Both P64 and P58 of B-TMEV(WW) appeared to have surface orientations by their reactions with iodine and whole virions and by their susceptibility to trypsin (Fig. 19). The similar radiolabeling characteristics of TC-TMEV(WW) suggested that its high molecular weight polypeptide, P64, also had a surface orientation (Fig. 20). The observation that B-TMEV(WW) was a consistent component of highly purified preparations of virus and could be reduced into two of the capsid proteins, P34 (VP2) and P27 (VP3) indicated that P58 was not a contaminant. The immunoprecipitation experiments confirmed that P58 was viral specific and the peptide mapping performed by limited proteolysis in gel indicated that P58 was the progenitor of

P27 (VP3) and P34 (VP2). These data suggested that P34 (VP2) and P27 (VP3) were linked together by a disulfide linkage. I am unaware of any reports detailing a large polypeptide in picornaviruses that can be reduced into two capsid proteins.

Only one large polypeptide was observed in preparations of TC-TMEV(WW). This polypeptide had a molecular weight of 64,000 daltons, and co-migrated with P64 of B-TMEV(WW) in SDS-polyacrylamide gels. The absence of P58 in preparations of TC-TMEV(WW) was the major consistent difference observed between B- and TC-TMEV(WW). Moreover, it was found that TC-TMEV(WW) P64 was not reducible when subjected to re-electrophoresis under reducing conditions like B-TMEV(WW) P58 (Fig. 21). Thus, these studies have defined one major biochemical difference between B- and TC-TMEV(WW). That difference was the absence of the 58,000 dalton molecular weight, apparently surface oriented, disulfide-linked precursor of capsid proteins VP2 and VP3 in highly purified TC-TMEV(WW). The origin of P44 in TC-TMEV(WW) electrophoresed under reducing conditions (see Fig. 20, lane C) remains unknown. This polypeptide was observed only occasionally in different preparations of TC-TMEV(WW), but was not a reduction product of P64 (see Fig. 21).

The occurrence of high molecular weight polypeptides in picornaviruses is not without precedence. A large polypeptide has been reported in foot-and-mouth disease virus (FMDV). FMDV contains a 52,000 dalton protein which maps to the 3'-end of the genome (283) and appears to be an in-

activated form of the viral replicase-polymerase (54, 253, 286). Because B-TMEV(WW) P58 was reducible into two of the major capsid proteins, it is very unlikely that P58 maps near the 3'-end of the TMEV(WW) genome, and therefore probably has no function as a polymerase or replicase. P58 probably represented a precursor of P34 (VP2) and P27 (VP3) which was present in populations of TMEV(WW). Preliminary peptide mapping of TC-TMEV(WW) P64 has provided suggestive, but not conclusive evidence that it is a precursor of P58; in that B-TMEV(WW) P58 peptide fragments are similar in mol wt to TC-TMEV(WW) P64 peptide fragments. Moreover, TC-TMEV(WW) P34 and B-TMEV(WW) P34 appear to be identical. The presence of high molecular weight polypeptides is probably analagous to the presence of trace amounts of VP0, the precursor of picornaviral capsid proteins, VP2 and VP4 (see Introduction, Fig. 1).

The presence of high molecular weight polypeptides in any TMEV strain examined was not reported in the study by Lipton, et. al. (176). The high molecular weight polypeptides reported in this study were probably detected because of the iodination radiolabeling conditions used. The high specific activities obtainable with iodination reactions (39) allowed detection of these polypeptides, which were present in relatively low concentrations in a given viral population.

The occurrence of the high molecular weight polypeptides P64 and P58 in a given viral population is unknown,

but may be due to ambiguous cleavage events occurring in an infected cell, or inefficient production or accessibility of post-translational processing proteases (see Introduction, "Structure, Antigenicity and Morphogenesis of Picornaviruses"). During lytic infection of a given cell type, normal production of capsid polypeptides VP1-VP4 would occur. If, however, in a certain proportion of cells, inefficient lytic infection ensued, ambiguous cleavage events (279) or ineffectual post-translational processing might occur and result in production of virions bearing precursor polypeptides. Given that both precursor-bearing and typical picornavirions existed in a given preparation of purified virus, electrophoresis of the entire population of virions would result in elucidation of both capsid and precursor polypeptides.

Because the results from preliminary peptide mapping experiments using limited proteolysis in gel indicated similarity between B-TMEV(WW) P58 and TC-TMEV(WW) P64, it may be likely that P64 is a precursor of P58. P64 was reducible <u>in vitro</u> in contrast to P58. This observation, coupled with the preliminary peptide mapping studies, suggest that the difference between P58 and P64 is that P64 probably contains a "joining" peptide fragment between the disulfide groups, which themselves, under reducing conditions are reduced into two sulfhydryl moieties. But under such conditions, the joining piece would prevent separation

of P64 into smaller polypeptides. P58, without the putative 6000 mol wt joining piece, would be reduced into two smaller polypeptides.

The fact that B-TMEV(WW) had both P64 and P58, and that TC-TMEV(WW) had only P64, suggested that two variants were present in B-TMEV(WW), and that tissue culture adaptation selected against the variant bearing P58. The possibility that P58 and P64 arise through ambiguous cleavage events or inefficient post-translational processing proteases, suggests that the BHK-21 C-13 cells in which B-TMEV(WW) was adapted to grow, were not permissive for the generation of P58 bearing variants. It seems unlikely that a random mutation event during tissue culture adaptation would produce TC-TMEV(WW) with P64. Alternatively, it is possible that the P64 bearing variants present in B-TMEV(WW) were capable of more efficient growth in cell culture and interfered with the growth of the P58 bearing variants. It is of little consequence whether P58 bearing virions were unable to replicate in vitro or whether the P64 bearing variants selectively interfered with the growth of P58 bearing virions, only P64 bearing viral particles were produced in tissue culture.

Biologically, B- and TC-TMEV(WW) differed in their ability to induce acute polioencephalomyelitis. Only B-TMEV(WW) was capable of inducing polioencephalomyelitis accompanied by flaccid hind limb paralysis. B-TMEV(WW) produced severe chromatolysis of neurons, neuronolysis and

induced an intense microglial response in brain and spinal cord gray matter during the acute phase of disease. B-TMEV(WW) was capable of inducing demyelinating and/or necrotizing myelitis in those animals which survived the acute phase of infection. In contrast, TC-TMEV(WW) had no capability of inducing the acute polioencephalomyelitic phase of disease, but was capable of producing chronic, progressive myelitis. Because B- and TC-TMEV(WW) differed biochemically in that only B-TMEV(WW) contained a percent of virions bearing P58, the possibility was raised that the presence of P58 bearing virions in the inoculum was related to the encephalitogenic potential of B-TMEV(WW).

To directly test the biologic significance of P58, B-TMEV(WW) (the only preparation which contained P58) was treated with a reducing agent, 2-mercaptoethanol (2-ME) to partially eliminate the P58-bearing virions from the inoculum. TC-TMEV(WW) was treated with 2-ME to assess the effect of chemical treatment on a population of virions which contained P64, but lacked P58. The ratio of 2-ME: virus was the same as that used in electrophoresis of whole B-TMEV(WW) under reducing conditions so that an assessment of the degree of reduction of P58 could be made. The results obtained indicated that marked reduction of P58 in the inoculum did produce a delay in the onset of morbidity and mortality, and significantly decreased the number of sick and dead animals at an early period of infection, but did

not completely ablate the encephalitogenic potential of the inoculum. These results were interpreted to indicate that 2-ME treatment provided for a partial reduction of viral titer, specifically due to the elimination of P58 bearing virions, since (i) P58 was the major polypeptide affected by 2-ME treatment of B-TMEV(WW), and (ii) identical experiments performed with TC-TMEV(WW), bearing only P64, did not result in any change in infectivity.

Two different assay systems were used to assess the effect of 2-ME treatment on TC- and B-TMEV(WW). 2-ME treated TC-TMEV(WW) was compared to untreated TC-TMEV(WW) in tissue culture to determine if any reduction in infectivity resulted from the chemical treatment of the variant bearing only P64. Treatment of TC-TMEV(WW) with 2-ME did not affect infectivity. Because the electrophoresis data indicated that TC-TMEV(WW) P64 was not altered when run under reducing or non-reducing conditions (viz a viz, B-TMEV(WW) P58), it was concluded that P64 was not required for infectivity during the first round of viral replication. 2-ME treated B-TMEV(WW) was compared to untreated B-TMEV(WW) in mice because B-TMEV(WW) could not be titered in tissue culture due to its inability to produce direct CPE. Care was taken to ensure that the aliquots of B-TMEV(WW) contained equal infectivities before 2-ME or buffer addition so that an assessment of the effect of 2-ME could be made. Any change in infectivity, measured by a delay in onset of clinical signs and death, would correlate with a direct effect of 2-

ME on B-TMEV(WW).

Specific reduction of viral titer due to partial decrease in P58 bearing virions in the inoculum probably caused a delay in the rapidity of viral replication, resulting in a depression of the degree of neuronolysis. Once, however, sufficient progeny virions had been produced *in vivo*, the infection could proceed unimpaired. If these interpretations are correct, they imply that P58 probably functions at the cell receptor level, rather than providing for more efficient intracellular replication. This interpretation is supported by the observation that P58 was a major contributor to the surface of B-TMEV(WW) as assessed by its reaction with iodine, preponderance in gels run under non-reducing conditions, and susceptability to trypsin. P58 may not play a direct role in the virus-cell attachment, as a receptor *per se*, but may impart a particular advantageous confirmational structure to the virion providing the P58 bearing particle a better orientation to attach to susceptable cells. Variations in confirmational structure correlating with antigenic conversion (i.e., D- to C- antigenicity) relating to the ability of a picornavirus to interact with susceptible host cell receptors has been discussed (see Introduction, "Structure, Antigenicity, and Morphogenesis of Picornaviruses").

The observation that tissue culture adaptation apparently selected against the variant bearing P58 from the original populations of virions bearing both P58 and P64,

raises the possibility that the late demyelinating disease produced in mice could be related to the P64 bearing variant. It is unlikely that a similar complete selection process occurs in vivo during infection, because persistent virus isolated from chronically ill animals retains its encephalitogenic potential when reinoculated intracranially into naive mice (167, Stroop, unpublished observations).

The observation that animals infected with TC-TMEV(WW) exhibited signs and histologic lesions much later than animals inoculated with B-TMEV(WW) suggests that the P64 bearing virus-host cell interaction might be less permissive than the P58 bearing virus-cell interaction and further suggests that the "relative-permissive, static-state model of Theiler's virus persistence may be valid (see Introduction, "Possible Mechanisms of Theiler's Virus Persistence and Chronic Disease Production"). In the case of TC-TMEV(WW) infection, the virus may attach, penetrate, and replicate in a relatively low permissive cell type. Virus replication would result in eventual lysis of the infected cell. Once sufficient cytolytic damage and antigenic stimulation of the infected mouse had occurred, the humoral immune response would tend to retard the rate of spread of the infection; virus would spread from cell to cell. The cellular immune response to infected and lysed cells would give rise to the characteristic appearance of lesions. The locations of the lesions almost exclusively in spinal cord white matter suggests that the relatively low permissive cell is one of

the cells common to the white matter, and not the neuron.

In the case of B-TMEV(WW) infection, the P64 bearing variant within the inoculum follows the same pattern as the P64 bearing variants of TC-TMEV(WW), however the P58 bearing variants may attach, penetrate and replicate in highly permissive cells, presumably gray matter neurons. Infection of these cells results in the characteristic chromatolysis, neuronolysis, and microglial proliferation of polioencephalomyelitis. If the infected animal can mount a humoral immune response sufficient to prevent its death, the rapidly replicated P58 bearing variants would be neutralized by antibody and thus prevented from spreading to other highly permissive cells. In the meantime, the P64 bearing variant's interaction with its relatively less permissive cell type could proceed, spreading progeny from cell to cell in the white matter. It may be because of the rapid rate of replication in highly permissive cells compared to the lower rate of replication in less permissive cells, that the differential effect of antibody present in the central nervous system of infected mice results in the two phase nature of disease following inoculation of B-TMEV(WW). The cellular immune response to the site of viral replication, in gray matter during the acute phase, and in white matter during the chronic phase, would account for the shift of the localization of the inflammatory response during the course of B-TMEV(WW) infection.

In this regard, it is of interest that Friedmann, et. al. (91), in examining the replication of the highly virulent GDVII strain compared to replication of the less virulent DA strain in vitro, noted that the DA strain produced few cell-free progeny, whereas the GDVII strain produced high levels of cell-free virus. These investigators also found that the DA strain virions were in a highly membrane associated state within infected cells, and formed few paracrystalline arrays; GDVII did not share the membrane affinity with the DA strain. Although these observations were made on two strains with vastly differing biologies in vivo (see Introduction, Tables 2, 3, and 5), they do suggest that the virus-cell interaction of the DA strain is less permissive than the GDVII virus-cell interaction.

The relative -permissive, static-state model of Theiler's virus persistence suggests that the neuron is the highly permissive cell type whereas the oligodendrocyte or other brain cell types are less permissive. If P58 bearing virions, which have been demonstrated by these studies to correlate with an ability to induce acute polioencephalomyelitis, preferentially replicate in neurons and the P64 bearing virions preferentially replicate in less permissive cell types, the distribution of viral RNA and antigens in central nervous system tissues following inoculation of B-TMEV(WW) should shift from predominately gray to exclusively white matter throughout the course of infection. Although it is clear that viral antigens are easily detected in gray

matter during the acute phase of infection, they are extremely difficult to demonstrate during the chronic phase of infection (167). The appearance of viral antigens following inoculation of TC-TMEV(WW) should be restricted to the white matter throughout the course of infection. Analysis of antigen distribution following inoculation of tissue culture adapted strains of Theiler's viruses have not been performed.

Localization of viral specific nucleic acids in infected cells during the course of a wide variety of viral infections including progressive multifocal leukoencephalopathy, visna virus infections of sheep, and herpes simplex virus infections (see Introductions, "Mechanisms of Viral Persistence in Production of Chronic Disease") have suggested that relative permissiveness of a given cell within an organ system to viral replication may be crucial to the pathogenesis of chronic disease.

If the relative-permissive static-state model of Theiler's virus persistence is correct, viral specific RNA should be detected in both cells of the white and gray matter shortly after infection of B-TMEV(WW). Viral RNA should be detected only in cells of the white matter following infection with TC-TMEV(WW). The RNA detected in cells of the white matter following B- or TC-TMEV(WW) infection would correspond with those cells infected with P64 bearing virions. The RNA detected in neurons during the acute phase of infection following B-TMEV(WW) infection would correspond to those cells infected with the P58

bearing virions. During the course of B-TMEV(WW) infection, RNA detected should shift from predominately gray (but also white) to exclusively white matter at about the time coincident with the rise in serum neutralizing antibody levels. Another prediction of the relative-permissive, static-state model would be that the number of copies of viral RNA found during the acute phase of B-TMEV(WW) infection would be greater in neurons than any other cell at any other time during infection, corresponding to the relatively high degree of permissiveness of neurons compared to the other cells of the central nervous system. Experiments of this nature would provide direct evidence for the validity of the relative-permissive, static-state model of Theiler's virus persistence in vivo, and provide further insight into the complexity of virus-host cell interactions which lead ultimately to the production of disease in the host.

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