IMMUNOLOGIC STUDIES ON PICHINDE VIRUS

IMMUNOLOGIC STUDIES ON PICHINDE VIRUS INFECTION IN CELL

CULTURE AND IN SYRIAN HAMSTERS

By

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ABSTRACT

Infection by Pichinde virus, a member of the arenavirus group, was studied _in vivo_ in Syrian hamsters, and _in vitro_ in cell culture. Emphasis in the _in vivo_ studies was placed on the mechanism of resistance to virus infection in adult hamsters. Two hamster strains were found to differ in their susceptibility to lethal Pichinde virus infection. LVG/Lak hamsters were found to be 100% susceptible to low doses of Pichinde virus during the first 6 days of life, but after 8 days of life, mortality was uncommon. Peak serum virus titers in animals infected at 3 days of life were 4 logs greater than in animals infected at 12 days. MHA/Lak hamsters, in contrast, were found to be susceptible to lethal virus infection both as newborns and as adults. Peak serum virus titers of greater than 10^8 PFU/ml were observed 8 days after infection of adult MHA hamsters compared with less than 10^3 PFU/ml in their LVG counterparts. Cultured primary kidney cells and peritoneal macrophages from either hamster strain supported Pichinde virus replication equally well _in vitro_. Antibody levels measured by complement-fixation test were

similar at 13, 21, and 30 days after infection.
Cyclophosphamide immunosuppression, administered 3
days after infection, rendered adult LVG animals
susceptible while slightly increasing the mortality
among MHA hamsters. These findings suggest that
immunologic factors maturing early in life in LVG
hamsters and deficient in MHA hamsters limit Pichinde
virus infection. The relationship of these observations
with previously reported arenavirus diseases is
discussed.

　　　The antigenic structure of Pichinde virus was
examined. Lysates of BHK_{21} cells, infected with
Pichinde virus and harvested 48-96 hours after infection
contained virus-specific antigens detectable by comple-
ment-fixation (CF) test. Immunodiffusion analysis
of the lysates demonstrated two antigens which differed
in their properties of heat and proteolytic enzyme
resistance. The antigen accounting for the major
proportion of the CF antigen activity was a heat stable,
pronase resistent protein of 20-30,000 molecular weight
estimated by gel filtration. The minor antigen was heat
labile and susceptible to proteolysis. Antiserum pre-
pared against partially purified major antigen, yielded
a band of identity when tested against antiserum

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prepared against virus "cores" obtained by NP-40

solubilization of purified Pichinde virus. Disruption

of purified virus by treatment with 1% NP-40 and 50

ug/ml RNase liberated 2 soluble antigens which

identified with those demonstrated in lysates of

infected cells. The liberated antigenic activity was

shown to contain 3 of the 4 polypeptides found in the

virion. These findings suggested that the antigens

detectable by CF and immunodiffusion were components

of the virion core. Major antigen derived from infected

cells was purified by rate-zonal sedimentation, iso-

electric focusing and gel filtration. Two low molecular

weight polypeptides were demonstrable in the purified

antigen.

Since multiple segments of RNA exist in the

Pichinde virion, it was of interest to determine

whether antigen synthesis and virus production could

be dissociated in the infected cells. In Vero cells

infected by Pichinde virus, antigen on the cell surface,

and production of infectious virus shut down 48-96

hours after infection, whereas antigen detectable in

the cytoplasm of infected cells appeared stable for over

5 days. In BHK$_{21}$ cells, actinomycin D (AD) at dosage

levels of 1-4 ug/ml reduced virus yields by greater than

95% while reducing antigen detectable by CF by only 30%.

The quantity of antigen produced was independent
of AD dosage within the range tested. Both the major
and the minor antigens were identified in lysates
of AD treated cells. The observed decrease in infectious
virus production could not be attributed to increased
cell associated virus or to greater production of
defective interferring particles. Surface antigen,
demonstrable by immunofluorescence and by antibody
binding assay was found to accumulate on the membrane
of infected cells incubated with AD. These findings
suggest that Pichinde virus replication in AD treated
BHK_{21} cells is blocked at the level of virus maturation.

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INTRODUCTION

I. Definition and Characteristics of the Arenavirus

The arenaviruses are a newly described group of viruses which are unique. Several members of the arena-virus group were formerly classed as arboviruses, but were reorganized as a separate virus group in 1970 when it was recognized that they possessed biological and structural properties distinct from the arboviruses (Rowe et al, 1970a). Rowe and co-workers (1970a, b) defined the arenaviruses as enveloped, RNA containing viruses which shared a common group-specific antigen demonstrable by immunofluorescence and complement fixation (CF) tests, and which showed a similar, unique virion structure in thin section electron micrographs. Virions were pleomorphic in shape ranging from 50-300 nm and averaging 110-130 nm. The particle consisted of a dense envelope with closely spaced spike-like projections and an interior lacking an electron dense core, but containing variable numbers of 20-30 nm electron dense particles resembling host ribosomes. The presence of these granules was the most unique feature of the arenaviruses, and it was this feature which suggested the name, from arenosus, Latin, sandy. Within the cytoplasm of the infected cell were seen large, non-membrane bound aggregates of these granules in a matrix

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of virus antigen. Progeny viruses were released from
the cell by budding at the cytoplasmic membrane. The
budding process was preceded by a thickening of the
membrane with the appearance of spikes on its exterior.
Simultaneously, aggregation of several 20-30 mm granules
appeared on the interior of the membrane at the maturation
site (Abelson et al, 1969).

There are currently 10 members of the arena-
virus group. These include lymphocytic choriomeningitis
(LCM) virus first isolated by Armstrong and Lillie
(1934) from a patient who died of what was thought to
be a case of St. Louis encephalitis. The virus was later
found by Traub (1936) to be endemic in a mouse colony in
Princeton, N.J. The biological and immunologic character-
istics of LCM virus have been studied extensively and
it is considered to be the prototype virus of the group.

Lassa virus, the etiological agent of Lassa
fever in central Africa is only distantly related sero-
logically to LCM virus, but appears to be morphologically
identical (Speir et al, 1970).

The Tacaribe complex of viruses make up the third
major subdivision of the arenavirus group. This group
(reviewed by Johnson et al, 1973) includes Amapari, Junin,
Latino, Machupo, Parana, Pichinde, Tacaribe and Tamiami
viruses. Junin and Machupo viruses are the etiological

agents of Argentine and Bolivian hemorrhagic fevers, respectively (Parodi et al, 1958; Johnson et al, 1965a). The other Tacaribe complex viruses do not appear to be pathologic for humans in a natural setting. Field studies by Trapido and Sanmartin (1970), by Jennings et al (1970), and by Webb et al (1970) uncovered no evidence of infection by Pichinde, Tamiami or Parana viruses, respectively, among people living in the areas in which these viruses were isolated. A single report (Buchmeier et al, 1974) indicates that under laboratory conditions of high risk exposure to Pichinde virus human infections may occur, however, no specific disease could be associated with these infections.

A high degree of antigenic cross reactivity exists among members of the Tacaribe complex. Rowe and co-workers (1970a), Johnson et al (1965), and Webb and others (1970) have documented antigenic cross reactivity detectable by CF test and immunofluorescence with virtually all of the Tacaribe complex viruses. In the study by Webb et al (1970), the cross reactivity between Parana virus and other Tacaribe complex viruses was found to be more pronounced when Parana-immune sera were tested against heterologous antigens than when Parana antigen was tested against heterologous antisera.

Cross reactivity of Tacaribe complex viruses with LCM virus was shown by Rowe et al (1970a) using immunofluorescent staining and CF. The cross reactions were most readily observed by immunofluorescent staining of viral antigens in the cytoplasm of infected cells.

Casals and Buckley (1973), examined the antigenic relationships between Lassa virus, LCM and the Tacaribe complex viruses. By CF test, a low but significant degree of cross reaction between Lassa and LCM was observed. Cross reaction was detectable using either Lassa or LCM antigen with the opposite antiserum. When hyper-immune Lassa antisera were used, weak cross reaction was observed with Amapari, Junin and Tacaribe viral antigens, but the reaction was not detectable using immune sera from persons surviving Lassa fever.

II. Biophysical Properties

It has been difficult to clearly define the biophysical parameters of the arenaviruses. For example, the sedimentation coefficient of LCMV has been reported to vary from 76S (Pfau, 1965) to 470-500S (Pederson, 1970) while the sedimentation coefficient of Pichinde virus was found to be 300-325S (Ramos et al, 1972). Possible explanations for the apparent variations observed include differences in purity and homogeneity of virus preparations used in the different studies and

the reported instability of some of the arenaviruses.
The published biophysical properties of the viruses of
the group are summarized in Table I.

,Members of the arenaviruses appear to vary in
their stability, especially upon purification and storage.
Studies by several groups of investigators have shown
that the viruses are rapidly inactivated by heat
(Pfau and Camyre, 1967; Parodi et al, 1966; Webb et al,
1967; Mifune et al, 1971) and at pH values below 6.0.
At physiologic temperature and pH, loss of infectivity
is not excessive. Webb et al (1967) investigated
factors contributing to the stability of Machupo virus
both under conditions of storage and during manipulation
in the laboratory. They found that virus stability was
improved by the addition of protein supplements to the
medium. Similar findings have been reported by Pfau
and Camyre (1967) and by Pederson (1970) working with LCMV.

III. Biochemical Properties-Nucleic Acids

Studies of the nucleic acids of the arenaviruses
have yielded unique findings in that a significant portion
of the RNA is derived from the host cell. The nucleic
acids extracted from purified virus preparations have
been found to be single-stranded RNA and multiple species
of RNA have been demonstrated. For LCMV, RNA species with
sedimentation coefficients of 31S, 28S, 22S, 18S and 4-6S

TABLE I

SUMMARY OF THE BIOPHYSICAL PROPERTIES OF DIFFERENT
ARENAVIRUSES

Parameter and Method	Virus	Reference
Virion Size by EM		
60-280 nm	LCMV	Dalton et al, 1968
		Abelson et al, 1969
	Machupo	Murphy et al, 1969
50-150 nm	Amapari, Latino Tacaribe, Tamiami Parana, Pichinde	Murphy et al, 1970
	Lassa	Speir et al, 1970
	Junin	Lascano and Berria, 1969
Sedimentation Rate in Sucrose Gradients		
470-500S	LCMV	Pederson, 1970
300-325S	Pichinde	Ramos et al, 1972
76S	LCMV	Pfau, 1965
Bouyant Density in Sucrose		
1.18 gm/cm^3	Pichinde Amapari LCMV	Mifune et al, 1971 Johnson et al, 1973 Pederson, 1973a
1.17 gm/cm^3	Junin, Machupo, Tacaribe	Johnson et al, 1973
1.12 gm/cm^3	Junin	Coto et al, 1972
Bouyant Density in Amidotriazoate		
1.14 gm/cm^3	LCMV	Gschwender et al, 1975b

were reported (Pederson, 1973b). Similar species of RNA
were found in Pichinde virus (Carter et al, 1973a) and
more recently a 15S species of RNA has been described
(Farber and Rawls, 1975). Carter et al (1973a) demonstrated
by base composition and by methylation ratio that the
28S, 18S and 4-6S species were similar to ribosomal RNA
of host cell origin while the 31S and 22S species were
different. Pre-labelled cellular RNA was incorporated into
the virus and appeared in the 28S, 18S and 4-6S segments
(Pederson, 1973b; Carter et al, 1973b). No direct
evidence has been presented to determine the functional
requirements of the host cell derived RNA in the replication
of the virus. Carter et al (1973b) found that Pichinde
virus replicated in the presence of low concentrations
of actinomycin D; the concentrations used suppressed
ribosomal RNA synthesis of the host cell. Thus, virus
replication did not require newly synthesized 28S and 18S
RNA.

An estimate of the molecular weight of the
Pichinde virus genome based on sensitivity to gamma
irradiation has been made (Carter et al, 1973b). The
radiosensitive genome was found to be 6-8 X 10^6 daltons,
greater than 3.2 X 10^6 daltons estimated from biochemical
data (Carter et al, 1973a), suggesting that the host RNA
in the virion may be functional. Single hit kinetics

of inactivation by UV light were found for Pichinde
virus which is compatible with only a single genome
copy per virion.

The question of whether the electron dense
granules observed in arenavirus virions are indeed
ribosomes has been the focus of much debate. Farber
and Rawls (1975) have recently described the bio-
physical and biochemical properties of these structures
isolated from Pichinde virus. The particles display
sedimentation and bouyant density properties similar
to BHK_{21} cell ribosomes. Analysis of the RNA component
of these particles showed that they contain 28S and 18S
RNA species. Pederson (1975) has examined the ribosomes
and nucleoproteins of LCM virus. Under conditions of
detergent disruption in the presence of 0.5 M KCl followed
by rate zonal centrifugation in the presence of 0.3 M
KCl several nucleoproteins could be isolated from the
virion. The isolated nucleoproteins sedimented at
129S, 83S, 60S, and 40S. The 2 larger species contained
33S and 22S RNA while the 60S and 40S species contained
28 and 18S ribosomal RNA. The 60S and 40S structures
had properties of EDTA and RNase sensitivity identical
to those of 60S and 40S ribosomal subunits. These
observations taken with those of Carter et al (1973a, b)
and Pederson (1973) showing that the 28S, 18S and 4-6S

RNA segments are of host cell origin make a strong
case for identification of the electron dense particles
as ribosomes derived from the host cell.

The mode of replication of the RNA of arenaviruses
has not been extensively studied. Previous work (Mifune
et al, 1971; Buck and Pfau, 1969) has shown that the
replication of several arenaviruses was inhibited by
the transcription inhibitor actinomycin D at levels
greater than 1 ug/ml. Carter et al (1974) detected
RNA-dependent RNA polymerase activity associated with
Pichinde virus, but no RNA-dependent DNA polymerase
was detected. Neither actinomycin D nor DNase inhibited
the endogenous polymerase reaction, but the reaction was
ablated by RNase indicating that endogenous viral RNA
served as a template for the polymerization reaction.
The polymerase required Mg++ and Mn++ for optimal
activity. The product was found to be partially resistant
to RNase, and the RNase resistant portion had a sed-
imentation coefficient of 22-26S.

Studies by a number of groups (Martinez-Segovia
and Grazioli, 1969; Coto and de Vombergar, 1969; Buckley
and Casals, 1970; Webb, 1965; Webb et al, 1967; Webb et al,
1970; Pfau et al, 1965; Mifune et al, 1971) have shown
that arenavirus replication is not inhibited by the
halogenated uridine derivatives bromodeoyuridine (BUDR)

and iododeoxyuridine (IUDR). Since these drugs inhibit
DNA replication, arenaviruses are presumed not to
require DNA synthesis for viral RNA replication. The
RNA inhibitor 6-azauridine has been shown to effectively
block LCMV replication (Buck and Pfau, 1969); thus
showing that RNA synthesis is necessary.

The host cell does not, however, appear to
function only as a substrate for virus-directed RNA
synthesis and translation. A recent study by Banerjee
et al (1976) has shown that Pichinde virus is unable to
replicate in cells enucleated with cytochalasin-B.
Enucleation up to 8 hours after infection was found to
be effective in preventing virus replication. The
mechanism of the block was not clear, however, it in-
volved complete inhibition of viral antigen synthesis.
This data taken with the inhibition of virus production
but not antigen synthesis by actinomycin D suggests that
replication depends to some extent on transcription of
the host cell genome. Whether the virus actually
requires a functional RNA synthetic system, or simply
needs a cell-coded protein cannot be resolved at this time.

IV. Biochemical Properties-Proteins

The polypeptide composition of Pichinde virus was
examined by Ramos et al (1972). Four polypeptides were
identified by electrophoresis of SDS disrupted virus in

polyacrylamide gels. Two polypeptides with estimated
molecular weights of 72,000 and 12,000 daltons were
found and called V_I and V_{IV}, respectively. Two gly-
coproteins, which were called V_{II} and V_{III}, were found
to have molecular weights of about 72,000 and 34,000.
daltons, respectively. The glycopeptide V_{III} was found
to be solubilized from the virion with the nonionic
detergent NP-40. Nonionic detergent treatment did not
dissociate V_I and V_{II} from the nucleic acid of the
virion while the fate of V_{IV} could not be determined
in the experiments. It was concluded that V_{III} re-
presented an outer membrane component. Pederson (1973a)
has reported the presence of 5 polypeptides in the LCMV
virion but a detailed account of the experiments has not
been published.

The proteins associated with infections by
the arenaviruses have usually been examined with reference
to their antigenicity. Smadel et al (1939, 1940)
reported the presence of a soluble complement-fixing
antigen in homogenates of guinea pig tissues infected
with LCMV. This antigen was separable from the virus
by ultracentrifugation. Repeatedly washed virions
reacted poorly in CF tests while the soluble antigen lost
none of its activity after ultracentrifugation.

Chastel (1970), Simon (1970) and Bro-Jorgensen (1971) have found antigens detectable by immunodiffusion in tissue or cell cultures infected with LCMV. Bro-Jorgensen found two antigenic species detectable by immunodiffusion using extracts of infected BHK_{21} cells as an antigen source. One antigen was found to be heat stable and resistant to pronase while the other antigen was found to be heat labile and susceptible to the proteolytic action of pronase. Both antigens sedimented at approximately 3.5S in sucrose gradients.

Recent studies by Gschwender (1975) have established that the extractable complement-fixing antigen (ECFA) of LCM virus-infected cells is an internal component of the virion. Antiserum directed against ECFA did not neutralize infectious LCM virus, nor would it mediate complement-dependent lysis of LCM virus-infected cells. Purified LCM virus disrupted by detergent treatment liberated an antigen which reacted in CF tests with anti-ECFA and which yielded a band of identity with ECFA by immunodiffusion.

Work contained in this thesis, and previously reported elsewhere (Buchmeier and Rawls, 1974, 1975) has led to similar conclusions. Antiserum directed against partially purified complement-fixing antigen of Pichinde virus obtained from infected cells has been

shown to react with internal components of the virus

but not on the surface of the infected cells. This

antiserum reacted, in immunodiffusion, with components

of NP-40 disrupted virions. The precipitin bands

obtained were antigenically identical with those

detected in lysates of infected cells.

V. Persistent Infections by Arenaviruses

Arenaviruses can, under certain circumstances,

set up either a persistent or an acute infection in vivo.

A classic example for virus persistence is chronic LCM

virus infection of the mouse originally described by

Armstrong and Sweet (1939) and by Traub (1936). Mice

infected in utero or neonatally with LCM virus typically

harbor virus in virtually all tissues and in blood and

excrete virus in the urine and saliva with no apparent

ill effects for life. This observation led Burnet

and Fenner (1949) to propose the theory of immunological

tolerance. The mice were thought to be, by virtue of

their early exposure to the virus, immunologically

unreactive or "tolerant" to viral antigens. In this

tolerant state the mice were assumed to co-exist

peacefully with infectious virus.

Oldstone and Dixon (1969, 1970a, b) proved that

mice persistently infected by LCM virus were not tolerant to

the virus. Upon careful examination these workers were able

to demonstrate virus-antibody complexes in the blood,
and were able to elute anti-LCM antibody from the
kidneys of chronically infected mice. Persistent LCM
infection was associated with a chronic glomerulo-
nephritis and arteritis due to the deposition of antigen-
antibody complexes on the basement membranes of the
glomeruli and blood vessels.

A marked genetic factor in the severity of the
immune complex glomerulonephritis was observed by
Oldstone et al (1973). Working with several inbred
mouse strains, these workers found that the severity of
the late disease was governed by the strength of the
anti-viral immune response mounted by the animal.
The SWR/J strain of mouse, which mounted a strong
anti-LCM response suffered more severe immune complex
disease than the C3H/HeJ strain which mounted a weak
response. The factor controlling this differential
response was shown to be linked to the H-2 locus.

Similar, though not as extensively studied, examples of
persistent infections have been reported for other
arenaviruses. These include Machupo virus infection of
the cricetine rodent Callomys callosus described by
Justines and Johnson (1969). Neonatal infection of
C. callosus by Machupo virus was shown to result in a
persistent infection with viremia and viruria lasting over

a year. Tamiami virus was shown by Jennings and co-workers (1970) to persist in cotton rats (<u>Sigmodon hispidus</u>) trapped in the Everglades region of Florida. Cotton rats infected in the first few days of life became persistently infected, but the infection in adults was readily resolved. Murphy and co-workers (1976) have studied the pathogenesis of Tamiami virus infection in newborn cotton rats and have demonstrated that viral antigens persist long after the disappearance of infectious virus from the tissues. In the brain, for example, infectious virus was cleared by 40 days after infection, but viral antigens were continually demonstrable in the cytoplasm of neurons for the duration of the 360 day study.

Pichinde virus persistently infects the cricetine rodent <u>Oryzomys albigularis</u> (Trapido and Sanmartin, 1971). Rodents trapped in the wild and held in captivity for from 111-455 days showed prolonged viremia in the presence of complement-fixing but not neutralizing antibody. Pichinde virus is apparently intimately associated with <u>O</u>. <u>albigularis</u>. Of 220 <u>O</u>. <u>albigularis</u> animals tested, 43 (20%) were positive for virus whereas only 1 of 988 animals of 15 species other than <u>O</u>. <u>albigularis</u> was positive for Pichinde virus. Similar host-virus relationships exist for other Tacaribe group arenaviruses (reviewed by Johnson <u>et</u> <u>al</u>, 1973).

VI. <u>Acute Disease Following Arenavirus Infection</u>

A striking contrast to the relatively benign host-virus relationship in persistent infection is seen in acute arenavirus infections. Acute infections have been reported in the natural host species for LCM virus and in unnatural hosts for LCM, Lassa, and many of the Tacaribe group viruses.

Acute infection in the natural host is best considered with reference to LCM virus disease in the adult mouse, <u>Mus</u> <u>musculus</u>. Inoculation of the adult mouse results in a severe disease which is usually fatal (reviewed by Hotchin, 1962; Lehmann-Grube, 1971). Lillie and Armstrong (1945) studied the pathology of acute LCM·infection in the mouse and found generalized lymphocytic infiltration in the viscera and brain. Kidneys, liver, salivary glands, pancreas, lungs, and adrenals were found to be heavily infiltrated with large lymphocytes and macrophages after LCM virus infection. In the central nervous system, the meninges, choroid plexus, ependyma, and spinal ganglia were affected. Maximum infiltration was found between days 6 and 9 after infection and varied somewhat with the route of inoculation.

Wilsnack and Rowe (1964) studied the histopathogenesis of acute LCM disease by immunofluorescence. Virus-specific antigen was found primarily in the liver

parenchyma, splenic reticulum, bronchi, and alveoli
after intraperitoneal inoculation. After intra-
cerebral inoculation, viral antigen was found pri-
marily in the meninges, choroid plexus and ependyma.
Antigen was never observed in the neurons in this
study.

Damage in acute LCM disease is due to a cell-
mediated immune response directed at virus infected
tissues. Evidence for this phenomenon comes from
several quarters. Hotchin and Weigand (1961) de-
demonstrated that pre-treatment of mice with immuno-
suppressive doses of x-ray precluded the development of
the typical LCM syndrome, but had no effect on virus
replication. Gilden and co-workers (1972a) utilized
cyclophosphamide immunosuppression to induce persistent
LCM infection in adult mice. Cyclophosphamide treatment
3 days after infection prevented the development of the
infiltrative disease, and yielded chronic carriers with
high brain and blood virus titers for periods exceeding
220 days. Reconstitution of such drug-induced carrier
mice with spleen cells from LCM-immune mice initiated
acute LCM virus disease (Gilden et al, 1972b). These
workers observed that the development of fatal chorio-
meningitis after infection was determined by multiple
factors including the distribution of viral antigen in the
carrier, level of circulating antigen, and time of cell

transfer after initiation of viral persistence. They were unable to initiate LCM disease by infusion of LCM carriers with hyperimmune anti-LCM antiserum.

Oldstone and Dixon (1970a) supplied direct evidence that acute LCM disease is caused by an anti-viral immune response. Chronic LCM carriers were exposed to LCM-immune lymphoid cells either by cell transfer from or parabiosis with hyperimmune syngeneic donors. Both procedures resulted in the rapid development of acute LCM disease in the chronic carriers. Transfer of anti-LCM antibody to chronic carriers resulted in acute necrotizing inflammatory lesions in sites of viral replication.

Acute LCM disease occurs in other species of rodents and in man. Löhler et al (1973) demonstrated widespread inflammatory lesions in the brains of young adult rats infected 5-6 days earlier with the WE strain of LCM virus. Guinea pigs have been utilized by some workers as a model for acute LCM-virus disease (reviewed in Lehmann-Grube, 1971).

Rivers and Scott (1935) first isolated LCM virus from a case of aseptic meningitis in a human, and many similar reports have followed (Armstrong and Sweet, 1939; Scott and Rivers, 1936; Smadel et al, 1942; Scheid et al, 1956; Baum et al, 1966). In humans,

the symptoms range in severity from a mild influenza-
like illness (Baum et al, 1966) to a fatal, febrile
meningitis (Smadel et al, 1942). In man, as in the
adult mouse, the most devastating damage appears to be
.the result of the host's own immune response against
virus-infected tissues.

Acute hemorrhagic fevers in man are caused
by two of the Tacaribe complex viruses. Junin and
Machupo viruses are the etiological agents of Argentine
(Parodi et al, 1958) and Bolivian (Johnson et al, 1965a)
hemorrhagic fevers, respectively. These diseases are
severe febrile illnesses marked by hemorrhagic diathesis
(reviewed by Johnson et al, 1973). Histologically,
there are relatively few specific lesions. Small focal
hemorrhages are seen in the tissues, and periodic acid-
Schiff positive inclusions are present in liver cells.
The production of a similar illness in laboratory
animals by Junin and Machupo has been unsuccessful with
one exception. The XJ strain of Junin virus causes a
hemorrhagic fever-like disease in guinea pigs (Boxaca
et al, 1961).

Lassa fever virus is responsible for a severe
disease characterized by myositis, myocarditis, pneumonitis,
kidney involvement and hemorrhagic diathesis. The disease
occurs in the West African countries of Nigeria,

Liberia, and Sierra Leone (Casals and Buckley, 1973).
The disease is severe and case fatality rates as high
46% have been recorded in some outbreaks. Epidemiological
evidence (Fraser et al, 1974) suggests that the disease
is less severe among members of the general population
in Sierra Leone than indicated from the previously
reported outbreaks. In these studies 6% of the populations
surveyed had complement-fixing antibody against Lassa
virus, but only 0.2% reported recognized disease.
With Lassa virus as with Junin and Machupo viruses,
there is no suitable animal model which reproduces
the human disease.

Pichinde virus has been found to infect humans
exposed in the laboratory (Buchmeier et al, 1974).
Antibody detectable by complement fixation was found in
46% of those workers exposed to concentrated virus in
the laboratory. No specific illness was attributable
to Pichinde virus infection under these conditions.

A fundamental difference exists in the patho-
genicity of the Tacaribe complex viruses and that of
LCM virus for newborn mice and hamsters. While LCM
virus infects the newborns and sets up a persistent
infection, viruses of the Tacaribe complex are lethal
for newborn hamsters and mice. The basis for this
difference is not well studied, however it can be

inferred that the lethality of the Tacaribe viruses
may be due to viral pathology and not due to immuno-
pathological damage.

VII. Immunosuppressive Effect of Arenavirus Infection

Infection by LCM virus is accompanied by
immunosuppression of the host. Work by Bro-Jorgenson
and Volkert (1972a) described "hemopoietic defects"
manifested by an increased sensitivity to x-ray in
LCM virus-infected mice. In a subsequent report,
Bro-Jorgenson and Volkert (1972b) showed that the
defect was traceable to a deficiency in hemopoietic
colony-forming stem cells in the bone marrow of LCM
virus infected mice.

Oldstone and co-workers (1973) examined the immune
responsiveness of mice, chronically infected with LCM
virus, to a variety of soluble protein antigens and to
sheep red blood cells (SRBC). Chronically infected
mice were shown to have normal immune responses to SRBC
and to keyhole limpet hemocyanin over wide dose ranges,
whereas their response to mammalian and avian IgG was
depressed. These workers traced the deficiency to de-
creased number of antibody forming cells in the spleens
of LCM virus-infected mice. No differences were noted
in the avidity of the antibody produced or in the
ability of peritoneal macrophages to process antigen.

Bro-Jorgenson and Volkert (1974), studying mice acutely infected by LCM virus, demonstrated that virus infection is followed by a period of general immunosuppression which developed in the second week after infection and persisted for 2-3 months. Hemopoietic colony forming stem cells were inhibited early in the infection, and thymus cells decreased dramatically during this period. These defects were surmised to be the cause of the immunosuppression. Although pre-existing antibody forming cells were not affected in acutely infected adult mice, in newborns the development of immune response to SRBC was transiently abolished. These authors suggest that this immunosuppressive effect exerted by the virus plays an important role in the establishment of the persistent infection in neo-natally infected mice.

In a subsequent study, Bro-Jorgenson and co-workers (1975) have measured the plaque forming cell responses of LCMV infected C3H mice to a variety of T-cell dependent and T-cell independent antigens, and to allografted DBA/2 mastocytoma cells. Responses to T-cell dependent antigens were found to be reduced by 92-96% from 2-4 weeks after infection. In contrast, the antibody responses to Escherichia coli lipopoly-saccharide, pneumococcal polysaccaride and polyvinyl-

pyrrolidone, B-cell dependent antigens, were within
normal limits. Cytotoxic cell responses against allo-
grafted DBA/2 mastocytoma cells were measured in vitro
by ^{51}Cr release. Spleen cells from LCM virus-infected
C3H mice previously grafted with 5 X 10^6 mastocytoma
cells were found to be impaired in their ability to
lyse the mastocytoma cells in vitro. The most severe
suppression of cytotoxicity was observed during a period
between 15 and 20 days after virus infection, however
recovery was not complete until 49 days after infection.

Persistent infection of the cotton rat by
Tamiami virus has been studied extensively by Murphy
et al (1976). These workers observed that Tamiami
virus shows a marked lymphoreticular tropism early after
infection. Peak virus titers and concentration of viral
antigens demonstrable by immunofluorescence were found
in lymph nodes, splenic white pulp, thymus, and bone
marrow early after neonatal infection of cotton rats with
Tamiami virus. Viral antigen reached peak concentrations
in these organs 16 days after infection. Peak antigen
concentration was reached in liver at 30 days, and in
kidneys, adrenal cortex, respiratory tract, and bladder
epithelium at 60 days; well after infectious virus had
declined in these organs. Production of virus antigen
in infected tissues was observed long after the dis-

appearance of infectious virus and the appearance of
neutralizing antibodies. The relationship between the
observed lymphoreticular tropism and virus-induced
immunosuppression is obscure at this time, however
infection of hemopoietic stem cells may well render
them incapable of normal maturation and differentiation,
the end result of which would be immunosuppression.

VIII. <u>Purpose of the Study</u>

The arenaviruses cause an important group of
virus diseases of man and animals. Their importance
lies not only in the immediate and serious public
health threat posed by the hemorrhagic fevers, but
also in their potential as models for the study of
persistent virus-host interactions <u>in vivo</u>.

The present studies were undertaken in order
to supply basic information into the structure and
replication of the arenaviruses, their antigenicity,
and their pathogenicity in laboratory animals. This
information will be essential in understanding the means
by which persistent infections are established and
maintained, and in determining the mechanisms involved
in the pathogenesis of both chronic and acute arena-
virus disease. It is hoped that the eventual un-
raveling of the puzzle posed by persistent infection will
contribute significantly to the understanding of chronic
and acute human disease of putative virus etiology.

MATERIALS AND METHODS

I. <u>Cell Culture and Virus Growth</u>

  A. <u>Cell Culture</u>

      BHK_{21} and Vero cell lines were grown in
stationary or rolling monolayer cultures in plastic
flasks (Corning). Cells were grown to confluency
using Eagle's minimal essential medium (MEM) supple-
mented with 10 mM glutamine, 0.075% $NaHCO_3$, 10 mM
hydroxyethylpiperazine N'-2 ethansulfonic acid pH 7.4
(HEPES), 100 units/ml penicillin and 100 μg/ml strepto-
mycin, and 10% heat inactivated fetal calf serum.
After infection, confluent monolayers were maintained
on MEM as above but with 0.15% $NaHCO_3$ and 2% fetal
calf serum.

      Primary kidney cells were cultured from minced,
trypsinized kidneys of three day old and adult hamsters.
The cells were seeded into 25 cm^2 plastic tissue culture
flasks and maintained on MEM supplemented with 10% heat
inactivated fetal calf serum. Flasks were sealed and
cultured at $37^{\circ}C$. Medium was changed after the first
24 hr of incubation, and cells reached confluency 2-3
days after seeding.

      Peritoneal exudate macrophages were obtained
from animals stimulated 72 hr earlier by intraperitoneal
(i.p.) injection of 5 ml of sterile mineral oil. Cells

were harvested aseptically by washing the peritoneal cavity repeatedly with Hank's balanced salt solution (HBSS). The harvested cells were washed twice with HBSS and counted in a hemocytometer. The cells were infected in suspension with 1-3 PFU of Pichinde virus per viable nucleated cell. Adsorption was for 60 min at $4^{\circ}C$ after which the cells were washed with, and resuspended in MEM supplemented with 10% heat-inactivated fetal calf serum. The resuspended cells were seeded into glass tissue culture tubes at a density of 2 X 10^6 cells per ml in a volume of 1 ml. Incubation was at $37^{\circ}C$ in an atmosphere of 5% CO_2.

B. Virus Source and Propogation

Pichinde virus strain AN3739 was originally isolated from the blood of an Oryzomys albigularis rodent in the Pichinde valley of Colombia by Trapido and Sanmartin (1971). The virus had been passaged 12 times in baby hamster brain prior to its receipt and was subsequently passaged 3 times in Vero cells and 4-6 times in BHK_{21} cells. Virus assays were performed on monolayers of Vero cells in 60 mm plastic tissue culture dishes as described elsewhere (Mifune et al, 1971). Virus and infected cells were obtained from monolayer cultures of BHK_{21} cells grown in disposable glass or plastic roller bottles. When confluent the monolayers

were infected at a multiplicity of infection (MOI)

of 1-3 plaque forming units (PFU) per cell by adding

2-4 ml of appropriately diluted stock virus to the bottle.

Adsorption was for 60 min at 37°C. Monolayers were then

washed once with phosphate buffered saline, pH 7.4

(PBS) and approximately 60-80 ml of maintenance medium

were added. The bottles infected in this manner were

incubated at 37°C with rolling at 1.25 revolutions/min

for 48-72 hr prior to harvesting virus and cells. Virus

was harvested and purified as previously described

(Ramos et al, 1972; Carter et al, 1973a; Rawls and

Buchmeier, 1975). Cells were washed once in situ with

borate buffered saline, pH 8.0 (BBS), then scraped into

cold BBS with a rubber policeman and washed twice more

by centrifugation. Following the second wash, the cell

pellet was resuspended at 20% vol/vol in cold BBS for

antigen extraction. Cell suspensions prepared in this

manner were stored at -50°C prior to use.

Isotopically labelled virus and cells were

prepared utilizing 5.0 uCi/ml ^{3}H-L-amino acid mixture

or 0.5 uCi/ml ^{14}C-L-amino acid mixture (New England

Nuclear Canada, Montreal). In some instances ^{3}H-

glucosamine-HCl (New England Nuclear, Canada; spec. act.

5-15 Ci/mmol) was used at a concentration of 5 uCi/ml to

label viral glycoproteins. The cells were overlaid

with maintenance medium containing the radiolabel 24 hr

after infection, and the virus and cells were harvested
48 hr later (72 hr after infection).

Actinomycin D (Calbiochem) was obtained in
lyophilized form and reconstituted with MEM prior to
use. Maintenance medium containing the drug at con-
centrations indicated in the text was added to virus-
infected cell cultures after adsorption. The culture
bottles were wrapped in aluminum foil prior to incubation
to prevent breakdown of the drug.

C. Soluble Antigen Extraction

Suspensions of infected cells prepared as
described above were frozen and thawed twice to lyse
the cells, then subjected to three 1 minute cycles of
sonication at maximum power (Bronwill Biosonik IV) in
a cup-type chamber with cooling on ice between cycles.

The sonicated lysates were then centrifuged
at 12,000 x g for 30 min and the pellet was discarded.
The supernatant material was used where indicated as
a source of antigen for immunodiffusion or complement
fixation (CF) tests. Where further clarification of
antigen was required, the supernatant from the 12,000 x g
centrifugation was centrifuged at 100,000 x g for 60
min and the pellet again discarded. The material re-
maining in the supernatant contained the soluble antigens
detectable by complement fixation and immunodiffusion.

II. Immunological Methods

A. Antisera

Immune sera against Pichinde virus were
raised in LVG/Lak strain golden Syrian hamsters and
in Hartley strain and random bred guinea pigs obtained
locally. The animals were infected by intraperitoneal
inoculation of 2.5 X 10^4 PFU of stock virus diluted
in Hank's balanced salt solution. The animals were
bled by cardiac puncture at 4 and 5 weeks after infection
and the sera obtained from these bleedings were pooled
and stored at -50°C in small aliquots. These sera will
be referred to as Pichinde-immune hamster or guinea pig
sera.

Hyperimmune antisera were prepared by immunization
of mice and hamsters with suspensions of virus-infected
suckling mouse or hamster brain, respectively. One to
two day old suckling mice and hamsters were infected
by subcutaneous inoculation of approximately 1000 PFU of
stock Pichinde virus, and their brains were harvested
8 days later when the disease was acute. Ten percent
w/v suspensions of homogenized brain were prepared in
saline and clarified by centrifugation of 2500 RPM for
20 min. The clarified supernantant was mixed at a 1:1
ratio with complete Freund's adjuvant (Difco) and
emulsified by repeated passage between two 10 cc glass
syringes linked by a double hub 18 ga. needle. Adjuvant

was judged to be satisfactory by placing a drop in

a beaker of water. If the drop did not spread, the

adjuvant was ready to use. This material was used to

immunize adult mice or hamsters according to the

following schedule:

> Day 0 - 0.1 ml of adjuvant in hind footpads
> (0.05 ml/footpad)
>
> Day 7 - 0.1 ml of adjuvant in the thigh muscle·
>
> Day 14 - 0.1 ml of adjuvant subcutaneously
>
> Day 21 - 0.1 ml of 10% brain suspension I.P.
> without adjuvant
>
> Day 35 - animals were exanguinated.

New Zealand white rabbits were immunized by

a single intravenous injection of 1 ml of purified

Pichinde virus. This dosage represented approximately

10^9 PFU of infectious virus. The rabbits were bled

weekly as described in Results.

Antiserum to partially purified antigen was

prepared by first processing the antigen as will be

described subsequently (Figure 1) and injecting the

partially purified material into guinea pigs. The peak

antigen containing fraction from an isoelectric focusing

gradient was dialyzed for 48 hr against several changes

of PBS and then emulsified in complete Freund's

adjuvant as described above. One half milliliter of this

material containing approximately 50 ug of protein was

```
          10% V/V SUSPENSION OF PICHINDE VIRUS
                 INFECTED BHK_{21} CELLS
                          |
                          v

               FREEZE THAW TWICE
                          |
                          v

          SONICATE - 3 1 MIN CYCLES
                          |
                          v

          CENTRIFUGE 60 MIN, 100,000 x g
                          |
                          |--------------------->  PELLET
                          v                          |
               SUPERNATANT                           v
                          |                       DISCARD
                          v

          CENTRIFUGE ON 5-20% PREPARATIVE
            SUCROSE GRADIENT - COLLECT
             FRACTIONS AND POOL THOSE
              CONTAINING ANTIGEN PEAK
                          |
                          v

          ISOELECTRIC FOCUS ON pH 3.5 - 10
            GRADIENT - POOL FRACTIONS
          CONTAINING HIGHEST ANTIGEN CON-
                    CENTRATION
                          |
                          v

              DIALYSE AGAINST PBS
                          |
                          v

            EMULSIFY IN COMPLETE
               FREUND'S ADJUVANT
                          |
                          v

          IMMUNIZE 3 GUINEA PIGS WITH 2
          DOSES EACH AT 14 DAY INTERVAL-
          BLEED OUT AFTER 24 AND 34 DAYS
                          |
                          v

               GUINEA PIG ANTISERUM
            AGAINST MAJOR (CF) ANTIGEN
```

Figure 1: Flow chart of methods used to obtain
          antiserum against partially purified
          Pichinde virus complement fixing antigen.

inoculated into several sites in the thigh muscles
of each of 3 guinea pigs. The animals were boosted
14 days later with a second dose. Ten and 20 days
following the final inoculation the animals were bled
by cardiac puncture.

Antisera were raised to components of the
virion. Appreciable quantities of virus were purified
as described by Ramos et al (1972). The purified virus
was divided into 2 aliquots at a 1:2 ratio and the smaller
aliquot was held on ice. The larger aliquot was
adjusted to a 0.2% Vol/Vol NP-40 (a gift of Shell Oil Co.),
vortex mixed, and incubated at $37^{\circ}C$ for 45 min. The
samples was then centrifuged for 2 hr at 100,000 x g
and the resulting pellet and supernatant were collected
and aliquots of all samples were tested for infectivity
in Vero cells. The untreated virus sample contained
in excess of 10^{10} PFU/ml while no infectious virus was
detected in the pellet or the supernatant of the
treated sample. The 3 samples were emulsified in
complete Freund's adjuvant and used to inoculate 3 groups
of 8 guinea pigs each. At 3, 6 and 9 days after inoculation
2 guinea pigs in each group were sacrificed and
samples of blood, liver and kidney were examined for
infectious virus. Only those animals receiving untreated
virus contained detectable amounts of infectious virus

in the blood.   The remaining 2 animals in each group were bled at 21, 28 and 36 days after inoculation.

B. Complement Fixation Tests

Complement fixation (CF) tests for detection of antigen or antibody were carried out in microtiter as previously described (U.S. Dept. H.E.W., 1969).   For detection of antigen a constant dilution of 1:100 of heat-inactivated ($56°C$, 30 min) Pichinde-immune hamster serum was used.   This dilution contained 5 or more CH_{50} units of complement fixing antibody.   Antigen endpoints were corrected for dilution factor inherent in the test procedure and the corrected values were used in calculation of antigen units per milligram of protein.

C. Immunodiffusion

Immunodiffusion was carried out on 25 X 75 mm microscope slides.   The slides were pre-coated with 1.0 ml of an 0.5% solution of purified agarose (Sigma) prepared in distilled water then dried.   Immediately prior to use the slides were coated with 2.5 ml of molten 1% agarose dissolved in BBS with 0.1% NaN_3.   Wells were punched using a template and reagents were added to the wells.   The slides were incubated for 72 hr at room temperature in a humid chamber, then washed for 5-7 days with daily changes of 0.15 M NaCl in the cold

followed by a 2 hr rinse in distilled water. The rinsed slides were then air dried and stained with 0.25% coomassie brilliant blue R-250 (BioRad) in methanol-water-glacial acetic acid (45:45:10). Destaining was carried out in the same solvent without dye for 15-30 min as required to clear background. Autoradiography on dried, stained ^{14}C labelled immuno-diffusion slides was done by attaching the slides to a sheet of Kodak No-screen^R x-ray film and exposing for 4 weeks.

D. Immunofluorescent Staining

Indirect immunofluorescence tests of sera were carried out on Vero or BHK_{21} monolayers grown on cover-slips. The monolayers were infected at a MOI of 1-3 PFU/cell and used 24-48 hr after infection. Coverslips were prepared for internal staining by thrice washing with PBS, air drying and then fixing the cells for 10 min in cold acetone. Cells were reacted with test serum for 30 min at $37^{O}C$ then washed three times and reacted with fluorescein isothiocyanate (FITC) conjugated goat-anti guinea pig IgG (Cappel Laboratories) for an additional 30 min at $37^{O}C$. Following the second incubation, the coverslips were washed twice with PBS and once with distilled water then mounted in buffered glycerol, pH 8.

Surface immunofluorescent staining of mono-
layers on coverslips was done by a previously described
method (Rutter and Gschwender, 1973). Briefly, mono-
layers of Vero cells were washed, reacted for 15-30
min with test serum at 20°C, then washed free of test
serum and fixed with ice cold phosphate buffered
formalin (pH 7.3) for 10 min on ice. Residual formalin
was washed off the coverslips and then FITC conjugated
goat anti-guinea pig IgG or rabbit anti-hamster IgG
was reacted with the cells for 30 min at 20°C. The
coverslips were then washed and mounted as above.

, Surface antigens were also detected using cells
which had been monodispersed with trypsin. The trypsin-
ized cells were washed once with PBS containing 2% fetal
bovine serum which had been heat inactivated, and aliquoted
into 12 X 75 mm glass tubes at 1×10^{5} cells/tube. The
cells were reacted for 30 min at 37°C with FITC conjugated
globulin from hamsters immunized with Pichinde virus,
washed 3 more times, and mounted in buffered glycerol on
microscope slides.

Direct immunofluorescent staining of acetone
fixed coverslip monolayers was performed using an FITC
conjugated globulin from Pichinde virus-immune hamsters.
The conjugated globulin was reacted with the cells for
30 min at 37°C. The coverslips were then washed three

times with PBS and mounted as above.

All immunofluorescent stains were examined using a Leitz Ortholux^R fluorescent microscope equipped with an HBO-200 mercury bulb, BG-38 and KP-490 excitation filters, and a K-510 or K-530 barrier filter. Observations were carried out utilizing reflected incident light. Photographic exposures of 1-3 min were made using Kodak Ektachrome^R film (ASA 160).

E. Antibody Binding Assay

Surface antigens were detected by ^{125}I IgG binding to viable, suspended cells. Hamster IgG was purified by 3 precipitations with saturated $(NH_4)_2SO_4$ at 50%, 40% and 35% saturation followed by ion exchange chromatography on a DE-52 DEAE cellulose column equilibrated with 0.05 M sodium phosphate buffer pH 7.4. The first protein peak eluted was shown by immunoelectrophoresis to contain only IgG. This peak was pooled and concentrated to 4 mg/ml protein. Aliquots of 40 ug were iodinated using the chloramine-T method of Greenwood et al (1963). The iodinated IgG was passed through a G-25 Sephadex column to eliminate unbound ^{125}I. The iodinated IgG was usually used without further dilution on the same day.

The cells were monodispersed with trypsin
and aliquoted into siliconized 12 X 75 mm tubes at
1 X 10^6 cells per tube. The cells were pelleted at
1000 RPM for 3 min, the supernatant drained from the
tubes, and 50 ul of ^{125}I labelled IgG added. After
30 min at 37°C the cells were washed 5 times with
PBS containing 2% heat inactivated fetal calf serum.
Before the final centrifugation, the resuspended cells
were transferred to new tubes and after the cells were
pelleted, the supernatants were removed and the radio-
activity remaining with the cells was determined using
a Beckman automatic gamma counter.

III. Biophysical and Biochemical Methods

A. Gel Filtration

Analytical gel filtration on Sephadex^R G-200
(Pharmacia) was carried out at room temperature in a
1.5 X 55 cm upward flow column eluted with BBS pH
8.0 containing 1:4000 NaN$_3$. The hydrostatic head
pressure was held constant at 11 cm of water and the
flow rate was 12 ml per hour. Void volume and total
volume of the column were determined by chromatography
of a mixture of 0.1% blue dextran (Pharmacia) and 0.04
mg/ml riboflavin (Eastman). Marker proteins of known
molecular weight (Schwarz-Mann) were utilized to calibrate
the column for molecular weight estimation. Antigenic
activity eluting from the column was determined by CF

test and by immunodiffusion.

Preparative gel filtration of material harvested from isoelectric focusing gradients was carried out using Biogel^R P-100 or P-200 columns (Bio-Rad). A 2.5 X 90 cm column was poured and equilibrated with BBS pH 8.0 with 1:2000 sodium azide. Four to five milliliters of pooled material was made 10% in glycerol and applied to the column. Elution was at a rate of 12 ml/hr. Antigenic activity was detected by CF and immunodiffusion.

B. <u>Density Gradient Centrifugation</u>

Rate zonal sedimentation was carried out in 5-20% w/v sucrose density gradients. The gradients were prepared in 0.1 M tris-hydroxymethyl amino methane (TRIS) buffer pH 7.4 and were formed with the aid of a gradient making device (Buchler). Centrifugation was for 20-22 hr at 35,000 RPM (SW-41 Beckman rotor or SB-283 IEC rotor) at 4°C. Fractions were collected by bottom puncture and the position of ^{125}I labelled protein markers was determined with a gamma counter. Marker proteins were also located in the harvested fractions by immunodiffusion using specific antisera against the individual marker proteins. Sedimentation values were estimated by the method of Martin and Ames (1961).

Preparative rate zonal sucrose gradients were utilized in purification of viral antigen. Twelve milliliter, 5-20% v/v sucrose gradients were made in cellulose nitrate tubes, and 1-2 ml of sample was layered on top of the gradient. The gradients were centrifuged for 22 hr at 35,000 RPM at a temperature of 4-5°C. Fractions were collected by bottom puncture and the antigenicity was determined in each fraction by complement fixation and immunodiffusion.

C. Isoelectric Focusing

Isoelectric focusing was done in a 110 ml glass column (Ampholine[R], LKB). Two percent Ampholyne[R] carrier ampholyte (pH 3.5-10) was used in a 0-46% w/v sucrose gradient. Samples were prepared for isoelectric focusing by dialysis overnight against 100 vol of 1% glycine at 4°C. Typically, 10-15 ml of dialyzed sample was used to replace an equal volume of water in preparation of the less dense sucrose solution. A gradient was formed using a mixing device (LKB) to mix the more dense (46% w/v) and less dense (0% w/v) sucrose solutions forming the sucrose gradient. Anode and cathode electrolyte solutions were 1% H_2SO_4 in 60% w/v sucrose and 2% ethylenediamine in water. For the broad pH range (3.5-10) gradient, voltage was regulated at 300 V for the first 24 hr then increased to 500 V for an additional

48 hr. Temperature was maintained at 5°C using a refrigerated circulator (Lauda K 2/R). At the termination of the electrofocusing run, the anode tube was plugged and the gradient pumped out at the rate of 80 ml/hr with a peristaltic pump. Fractions of 2.5 ml were collected and the pH determined at 5°C using a Radiometer pH meter equipped with a microelectrode.

Viral proteins were liberated from purified virus disrupted by treatment with 2% NP-40, ethyl ether and RNase (50 ug/ml). Typically, a solution containing 1-3 mg of purified virus protein was adjusted to 2% in NP-40 then extracted twice with 4 vol of anhydrous ethyl ether. After blowing off the residual ether with nitrogen gas, ribonuclease-a (Sigma) was added to a concentration of 57 μg/ml and the mixture was incubated for 30 min at 37°C. Disrupted virus obtained in this way was incorporated into a pH 3.5-10 gradient and electrofocused as described above with the exception that 0.1% NP-40 was added to the sucrose solutions.

D. Polyacrylamide Gel Electrophoresis

Polyacrylamide gel electrophoresis in gel columns was carried out as previously described (Ramos et al, 1972) using the discontinuous buffer system originally described by Laemmli (1970). For optimal separation of virion polypeptides, 15% acrylamide gels containing 0.2%

SDS and 0.5 M urea were used. Gels were sliced into
1 or 2 mm segments using a Gilson automatic gel
fractionator and the radiolabelled polypeptides were
eluted from the segments into 0.3 ml of solubilizer
(10% Beckman BBS-3 in water). After 4-12 hr at room
temperature, 8 ml of scintillation fluid consisting of
toluene, Fluoralloy-TLA^R (Beckman) and 10% BBS-3 was
added, and the fractions were counted in a liquid
scintillation counter. In gels where double labelling
was employed, ^{3}H-counts per minute were corrected for
spillover from the ^{14}C-channel, and the corrected CPM
plotted.

Slab gel electrophoresis was carried out using
the discontinuous SDS system described by Laemmli (1970)
without modification. Ten to fifteen percent gels were
electrophoresed overnight at a constant potential of
100 V at 22°C. Gels were stained at 0.25% coomassie
brilliant blue R-250 prepared in methanol, glacial
acetic acid, and water (25:7:68) for 2-3 hr at room
temperature then destained in the same solvent without
dye. Radioactivity was detected using the fluoroauto-
graphy method of Bonner and Laskey (1969). Exposures of
dried, PPO impregnated gels were carried out at -70°C for
7-14 days to detect ^{3}H and 4-7 days to detect ^{14}C. Films
were processed in a Kodak X-omat^R automatic processor.

E. Protein Determination

Protein concentrations were estimated by the method of Lowry et al (1951) using bovine serum albumin as a standard. Where direct performance of Lowry protein determination was impossible due to interferring components in the sample (i.e. electrofocusing ampholyte or NP-40), the protein was precipitated with 15% trichloroacetic acid (TCA) overnight on ice, and the precipitated proteins re-dissolved in 0.1N NaOH or 1% SDS and used for Lowry protein determination.

IV. In Vivo Biological Methods

A. Animals

Hamsters used in this study were obtained from Charles River/Lakeview. Strains used included LVG/Lak random bred and MHA/Lak, LHC/Lak, CB/Lak, PD-4/Lak and LSH/Lak inbred hamsters. Adult animals were obtained at 5-9 weeks of age and used at 6-12 weeks of age. Pregnant females were obtained at 12-13 days of gestation and litters usually were born on the 17th day. Newborns were used in the first 72 hr of life unless otherwise indicated. Suckling hamsters were inoculated subcutaneously on the back with 0.05 ml of virus dilution. Observations for illness and death were made daily starting on the second day after inoculation. All were housed in wire topped polycarbonate cages and fed Purina Chow and water ad lib.

Young adult BALB/C mice were obtained from Texas Inbred Mice. Pregnant female BALB/C mice were obtained several days prior to littering. The young were inoculated subcutaneously on the back with 0.05 ml of virus dilution.

B. Cyclophosphamide Treatment

Cyclophosphamide (Procytox[R], Horner Ltd, Montreal) was prepared at a concentration of 10 mg/ml in saline immediately prior to use. Hamsters were treated 3 days after virus infection by i.p. injection of 100 or 150 mg/kg of cyclophosphamide as indicated in the text. Non-infected drug control hamsters included in each experiment revealed no lethality due to cyclophosphamide treatment alone.

C. Tissue Virus Assays

Infectious Pichinde virus in the blood and organs of infected hamsters was quantitated by plaque assay (Mifune et al, 1971). Solid organs were weighed, then ground in a mortor with sufficient MEM to make a 10% w/v suspension. The suspension was clarified by centrifugation at 1500 RPM for 15 min, and the supernatant fluid was titered. Titers were expressed as PFU per gram of solid organs or PFU per milliliter of serum or blood. All samples were held at $-85^{\circ}C$ prior to titrations and groups of samples of a single organ were assayed in the same

titration.

V. Human Population Study

A total of 44 persons from the Departments of Virology and Epidemiology, and Microbiology, Baylor College of Medicine, Houston, Texas, were studied. Two or more serum samples were analyzed from 35 persons and single serum samples were analyzed from 9 persons. Based on their potential exposure to Pichinde virus, these individuals were categorized into risk groups. Individuals of high risk were those actually working with Pichinde virus, whereas individuals of moderate risk were those working in the same laboratory area but not with the virus. Low risk individuals were those working with viruses other than Pichinde in different laboratory areas of the same building. None of the persons studied were exposed to other members of the arenavirus group which share cross-reacting antigens. Sera were collected between March 1972 and November 1973. The single serum samples were all collected in October, 1973. All sera were coded prior to testing and the code was not broken until the results were recorded.

RESULTS

I. Susceptibility of Unnatural Hosts to Infection by Pichinde Virus

Initial surveys by Trapido and Sanmartin (1971) suggested that Pichinde virus was not hazardous to humans. In addition, the virus appeared to have a limited host range specificity in its natural ecological setting. To substantiate these observations, the immune responses of several laboratory animal species to Pichinde virus infection were examined.

A. Immunization of Laboratory Animals with Pichinde Virus

The first method of immunization which was attempted was that of intravenous injection of quantitities of purified Pichinde virus into rabbits. Antisera raised in this way were potent, and CF antibody titers in the range of 1/128-1/256 were obtained. A serious drawback of antisera raised in this way was their contamination with antibodies to components of BHK_{21} cells. Figure 2 shows the rise in virus specific and non-specific CF antibody titers in one rabbit (VO-105) after a single intravenous injection of purified Pichinde virus. The nonspecific antibody titer increases along with the specific titer. Immunodiffusion analysis

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Figure 2

       Complement-fixing antibody produced by a
rabbit after a single intravenous injection with
purified Pichinde virus. Antibody titers determined
against antigen extracted from virus infected
(O—O) and control (△—△) BHK_{21} cells are shown.

CF ANTIBODY, TITER

WEEKS AFTER INOCULATION
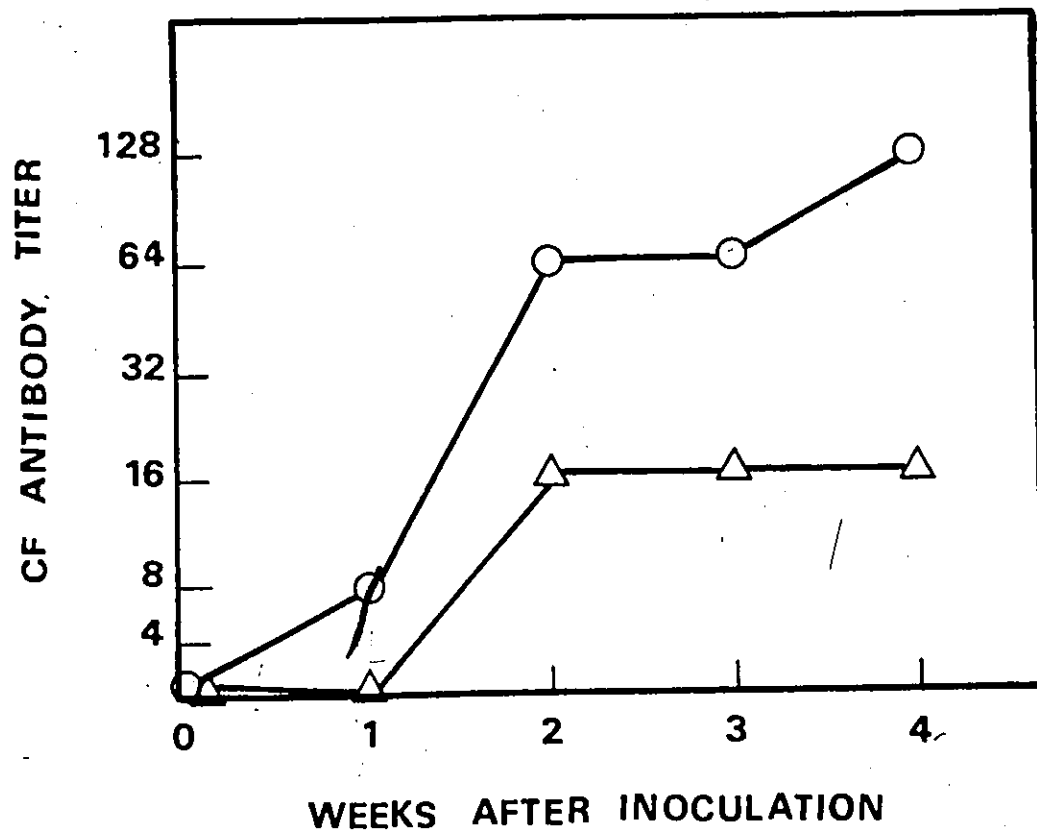
of the antiserum showed two precipitin bands which
were specific for virus infected cells, and one cell
specific band. Indirect immunofluorescent staining of
Pichinde virus infected cells with rabbit antiserum
yielded a granular pattern of cytoplasmic fluorescence
superimposed on a diffuse background staining. Staining
of non-infected control cells yielded only the diffuse
background staining.

More specific, though not completely suitable,
antisera were raised by a method used extensively by
Webb and co-workers (1970). Newborn mice and hamsters,
both susceptible to lethal Pichinde virus infection,
were infected and allowed to become acutely ill. Brains
were removed 8 days after infection and a suspension
prepared and incorporated into complete Freund's
adjuvant as described in Materials and Methods. The
immunogens were used to inoculate adult mice and hamsters,
respectively, and sera obtained two weeks after the final
inoculation were pooled. Table II summarizes the results
of CF and immunodiffusion analyses of these sera.
Specific CF antibody titers were high, but the hyper-
immune sera were often anti-complementary in our hands.

A third, more successful approach to antiserum
production was that of direct infection of adult
hamsters and guinea pigs with $2 \times 10^4 - 1 \times 10^6$ plaque
forming units (PFU) of Pichinde virus grown in BHK_{21} cells.

TABLE II

ANALYSIS BY COMPLEMENT FIXATION AND IMMUNODIFFUSION
OF HYPERIMMUNE HAMSTER AND MOUSE ANTISERA RAISED BY
INOCULATION OF VIRUS-INFECTED HOMOLOGOUS BRAIN SUS-
PENSION IN ADJUVANT

| Source | CF Reactivity | | ID[1] Bands | |
	Virus-Specific[2]	Anticompl.[3]	Major	Minor
Hamster (Pool of 12)	1:2048	1:32	+(1:4)[4]	-
Mouse (Pool of 30)	1:160	1:4	+(1:8)	+(1:1)

[1] ID = Immunodiffusion bands when tested against a
single dilution of a soluble extract of virus
infected BHK_{21} cells.

[2] Titer against soluble extract of virus infected cells.

[3] Anticomplementary titer = Highest dilution of antiserum
fixing complement in the absence of antigen.

[4] + Denotes presence of immunodiffusion band. Number
in parentheses is highest dilution of antiserum
giving positive reaction.

Hamsters infected in this manner responded with the production of CF antibody which titered in the range of 1/512 to 1/1024 and occasionally higher. The antibody appeared between 7 and 14 days after infection and reached peak titers at 21-28 days (Figure 3). Control anti-BHK and anti-fetal calf serum tests were negative, and these sera were not anticomplementary at a 1:2 dilution. Sera obtained in this manner were routinely used at a dilution 1:100 for the detection of antigen by CF.

Immunodiffusion bands obtained using immune and hyperimmune hamster sera were not sharp enough for resolution of multiple antigenic species. For this purpose, Pichinde-immune guinea pig serum raised by a single infection of 2-3 X 10^4 PFU of tissue culture obtained virus was used. This serum, harvested 5 weeks after infection yielded two sharp virus-specific precipitin bands when tested against lysates of cells infected with Pichinde virus (Figure 4). These bands were shown by immunodiffusion to be identical to the virus-specific precipitin bands detected by the previously described rabbit antiserum.

Figure 3

        Complement-fixing antibody titers in adult

LVG hamsters after infection with Pichinde virus.

30 adult LVG hamsters were infected by intraperitoneal

 inoculation of 2.5 X 10^4 PFU of Pichinde virus, and

groups of 7-12 animals were bled and their

virus-specific CF antibody titers determined. Each

point represents an individual serum sample.

CF ANTIBODY TITER vs DAYS AFTER INFECTION
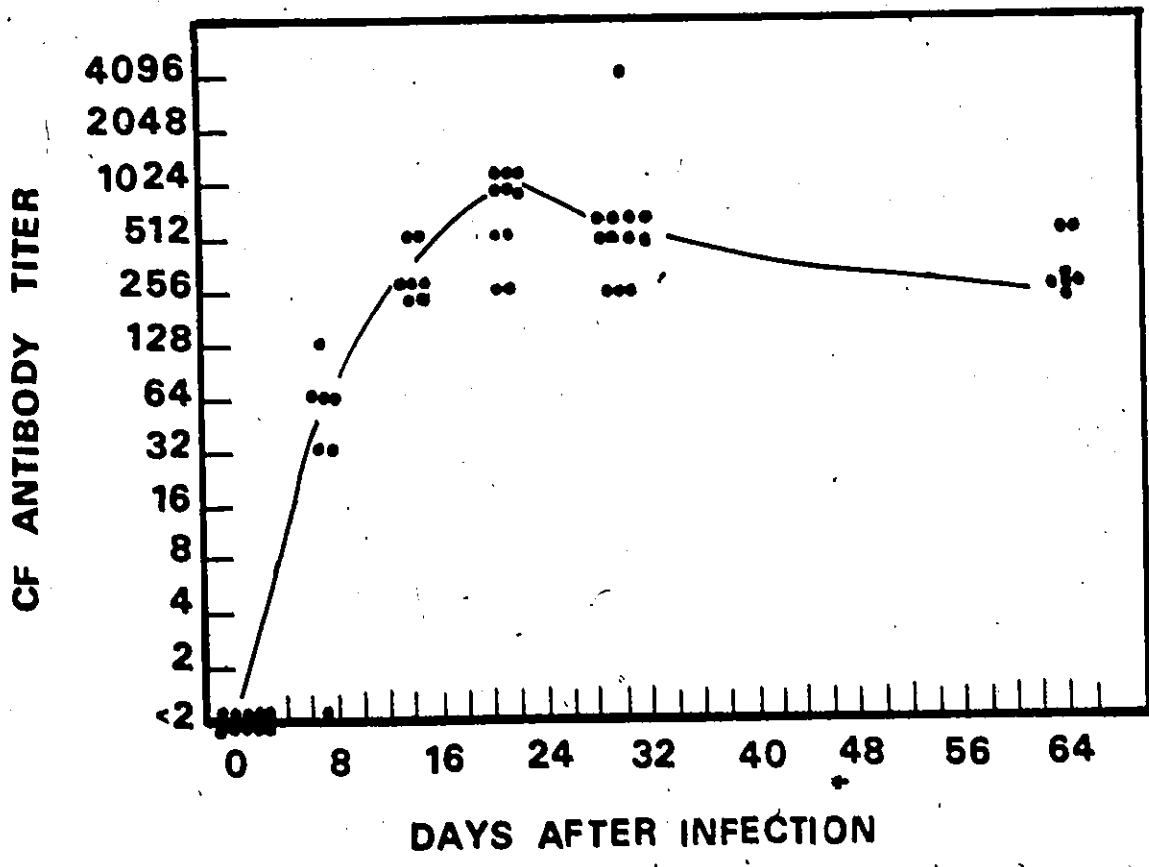
Figure 4

Immunodiffusion reaction between serum from Pichinde virus-immune guinea pigs and soluble antigen prepared from virus-infected and control BHK_{21} cells. The wells contain the following: (A) soluble antigen from virus infected cells; (AS) the peak antigen-containing fraction from a 5-20% sucrose density gradient centrifugation run of soluble antigen from virus-infected cells; (C) soluble antigen from non-infected BHK_{21} cells; (CS) the fractions corresponding to AS above, from a 5-20% sucrose gradient centrifugation run of control cell extract. The center well (S) contained antiserum from Pichinde virus-immune guinea pigs.

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B. Pathogenesis of Pichinde Virus Infection in Hamsters

  1. Susceptibility of LVG and MHA Hamsters to Lethal Infection by Pichinde Virus

Adult hamsters of the LVG and MHA strains were found to differ in their susceptibility to lethal infection by Pichinde virus. Animals of both strains were susceptible as neonates, but LVG hamsters became resistent to lethal infections between 6 and 8 days of age. Figure 5 shows mortality curves generated when LVG hamsters were infected at 0, 4, 6 and 8 days of age with 500 PFU of Pichinde virus. The animals were found to be uniformly susceptible if inoculated in the first 6 days of life, but resistent if inoculated later. MHA hamsters (Figure 6), in contrast, were found to be susceptible to virus doses as low as 35 PFU at 7 weeks of age.

Investigation of the acquisition of resistance in LVG hamsters revealed that with increasing age at the time of infection, virus replication was limited. Table III summarizes the virus titers reached in kidneys and brains of LVG hamsters 4 and 9 days after the animals had been infected with identical virus doses at 3 and 6 days of life. Note that without exception, the highest titers were reached in the youngest animals. Table IV shows

Figure 5

Mortality among LVG hamsters after Pichinde
virus infection at various ages. Hamsters were
infected by subcutaneous inoculation of 500 PFU of
Pichinde virus on days 0 (⊙—⊙), 4 (☐—☐),
6 (△—△), and 8 (◇—◇) after birth, then ob-
served daily for death.
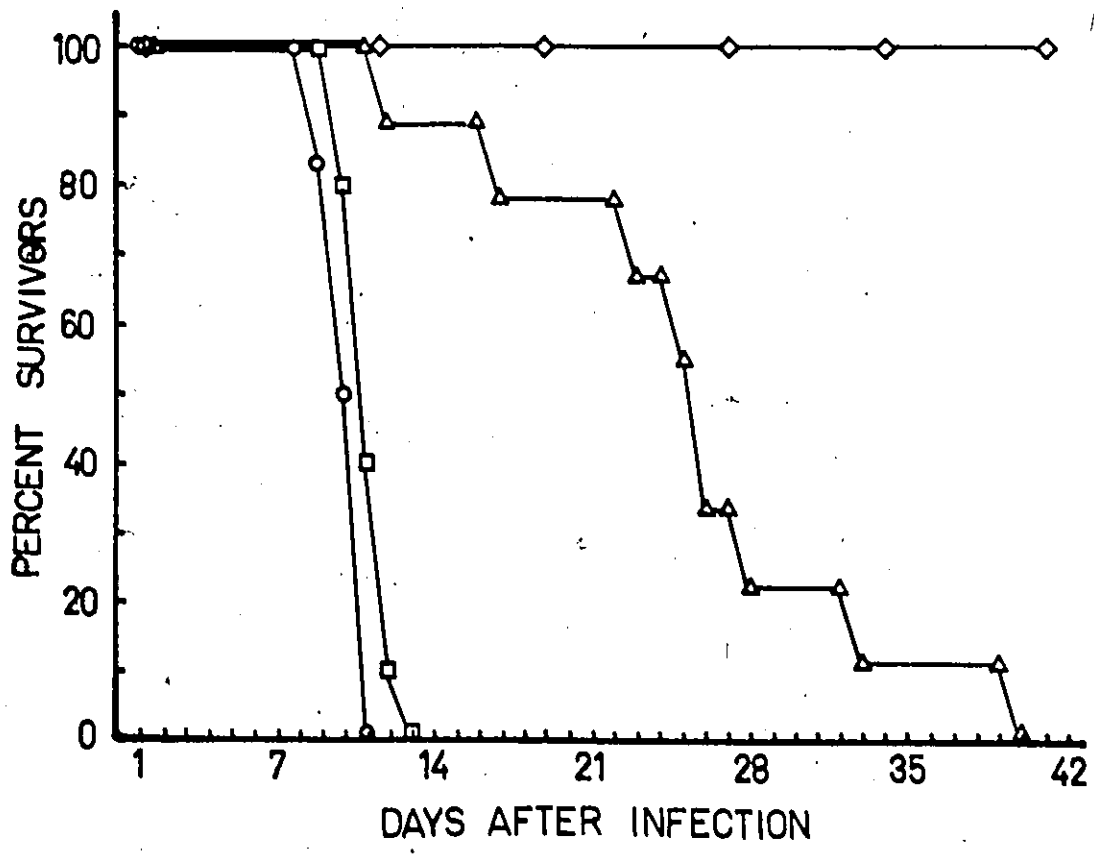
Figure 6

Mortality among adult MHA hamsters after intraperitoneal infection with various doses of Pichinde virus. Virus doses used were: (O—O) 3.5×10^6 PFU; (△—△) 3.5×10^4 PFU; (□—□) 35 PFU per animal.
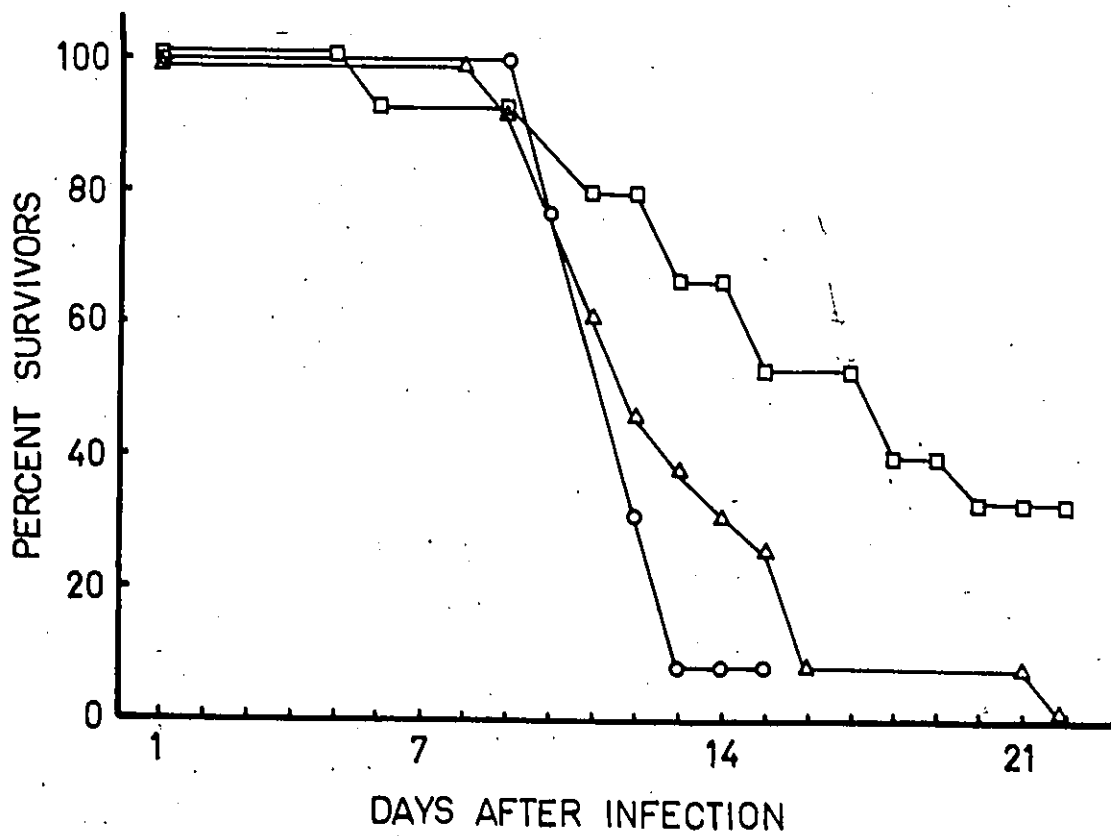
TABLE III

PICHINDE VIRUS TITERS IN KIDNEY AND BRAIN 4 AND 9

DAYS AFTER INFECTION OF LVG HAMSTERS AT 3 AND 6

DAYS OF AGE

Age at Inoculation (days)	Virus titer[1]			
	Day 4[2]		Day 9	
	Kidney	Brain	Kidney	Brain
3	6.2	3.3	7.1	7.25
6	4.9	<1	6.6	6.0

[1] Log_{10} virus titer in PFU/gram.

[2] Day after inoculation.

TABLE IV

SERUM TITERS OF PICHINDE VIRUS AFTER INFECTION OF LVG HAMSTERS AT VARIOUS AGES

	Virus titer[1]	
Age at Inoculation	Day 4[2]	Day 9
3 days	5.8	8.4
6 days	3.5	5.3
12 days	3.2	4.1
Adult (>30 days)	3.0	<3.0

[1] Log_{10} virus titer in PFU/ml.

[2] Day after inoculation.

blood virus titers in hamsters which were 3, 6 and 12
days old at the time of infection compared with animals
inoculated as adults. Again, the virus titer is highest
in the youngest animals and lowest in the adults. Titers
are seen to increase between 4 and 9 days after infection
in the 3, 6 and 12 day old animals, while decreasing during
this period in adults.

2. Virus Titers in Organs after Infection of LVG and
MHA Hamsters

A possible explanation for the increased
susceptibility of MHA hamsters is the failure of these
animals to successfully limit virus replication. To
examine this possibility, adult MHA and LVG hamsters
were infected with identical doses of Pichinde virus,
then virus titers in various organs were compared at
intervals after infection. Figure 7 illustrates the
kinetics of viremia observed in these animals. The serum
virus titers rose in parallel until 4 days post-infection
after which serum virus titers of MHA animals continued
to increase and became more than 5 logs greater than
those of the LVG animals at 8 days after infection. A
comparison of virus titers in various other organs of
the two strains of hamsters can be made from the data
presented in Table V. In all of the organs tested

Figure 7

Viremia in MHA and LVG hamsters after
Pichinde virus infection. The hamsters were in-
fected with 2 X 10^5 PFU of Pichinde virus intra-
peritonealy, and two animals were tested at each
time indicated.
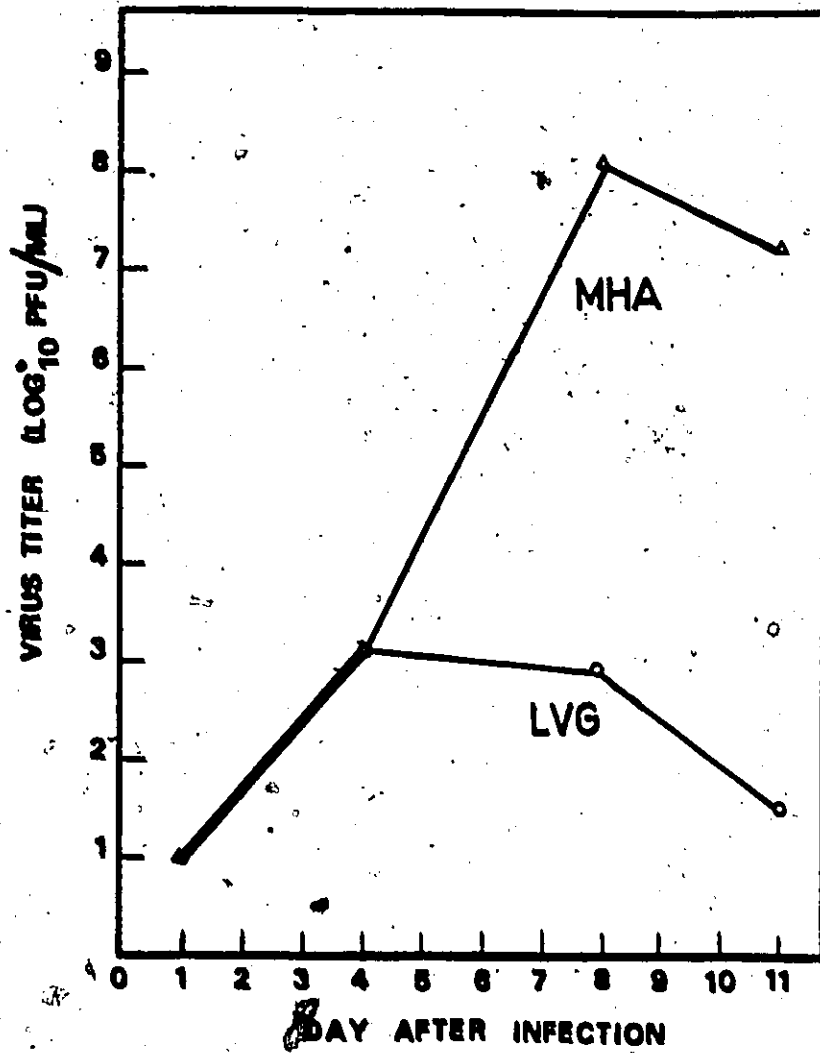
TABLE V

PICHINDE VIRUS TITERS IN VARIOUS ORGANS OF LVG AND
MHA HAMSTERS AFTER INFECTION

Organ	Hamster Strain	Day After Inoculation				
		1	4	8	11	15
Blood	MHA	0^1	3.1	8.1	7.2	NT^2
(serum)	LVG	0	3.1	2.9	1.5	NT
Kidney	MHA	0	$---^3$	6.7	8.4	≥ 8.0
	LVG	0	3.9	4.2	---	4.5
Spleen	MHA	0	---	8.0	8.1	8.4
	LVG	0	5.65	6.4	2.5	2.7
Brain	MHA	0	---	5.6	6.62	NT
	LVG	0	<2	<2	<2	NT

[1] Log_{10} virus titer in PFU/ml of serum or PFU/gram of solid organs.

[2] NT - Not Tested.

[3] --- Indicates no data available.

the peak virus titers reached in MHA hamsters were greater than those of their LVG counterparts.

3. Primary Cell Cultures from both Strains Support Virus Replication Equally Well

The difference in virus levels observed in the two strains could be explained by a difference in the ability of their respective cells to support virus replication. To test this hypothesis, primary cell cultures were prepared from the kidneys of 3 day old and adult animals of each strain, and growth curves of Pichinde virus were determined in each cell type. Figure 8 shows the results of these experiments. Kidney cells of either strain supported virus replication to similar levels. The overall levels of virus were slightly higher in the cells cultured from newborns of both strains than from adults; this may have been attributable to the greater number of cells in the newborn cultures (data not shown).

It was conceivable that a difference existed in the ability of macrophages to support virus replication. Peritoneal exudate macrophages were examined for their ability to support virus replication in vitro. Oil stimulated peritoneal exudate cells were harvested and cultured as described in Materials and Methods, and growth curves of Pichinde virus were performed. Figure 9 illustrates the results of one such experiment. Peritoneal

Figure 8

        Growth of Pichinde virus in primary kidney
cell cultures established from 3 day old and adult
MHA and LVG hamsters. Identical cultures established
from 3 day old and adult MHA and LVG hamsters were
infected with 1-3 PFU/cell of Pichinde virus. After
adsorption, cells were overlaid with maintenance
medium, and incubated. Medium was changed at 24 hour
intervals, and samples taken at the times indicated
represent virus produced in the previous 24 hours.
Symbols: (▲--▲) 3 day MHA; (●--●) 3 day LVG;
(△--△) adult MHA; (○--○) adult LVG.
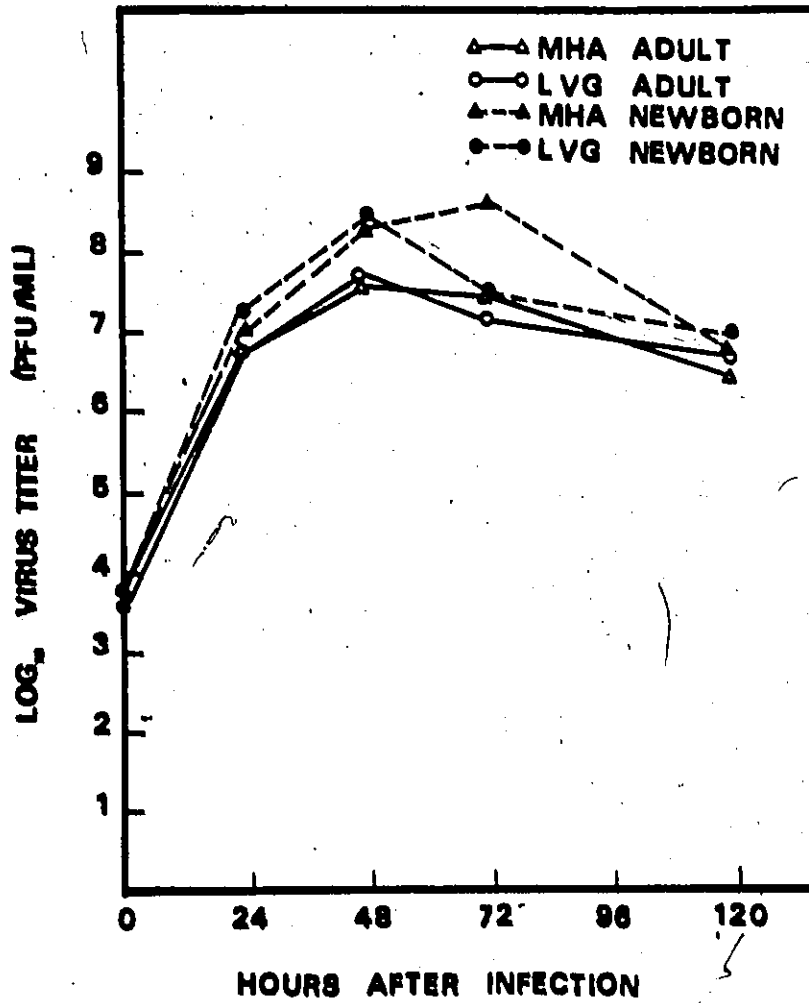
Figure 9

Growth of Pichinde virus in LVG and MHA hamster macrophages cultured in vitro. Peritoneal exudate macrophages were harvested 3 days after stimulation with sterile mineral oil and infected with 1-3 PFU/cell of Pichinde virus. After adsorption for 60 min at 4°C the cells were washed then seeded into culture tubes (2 X 10^6 cells/tube). Two tubes were removed at each time point, and total medium and cell associated virus was titered. (●—●) LVG macrophages; (O—O) MHA macrophages.
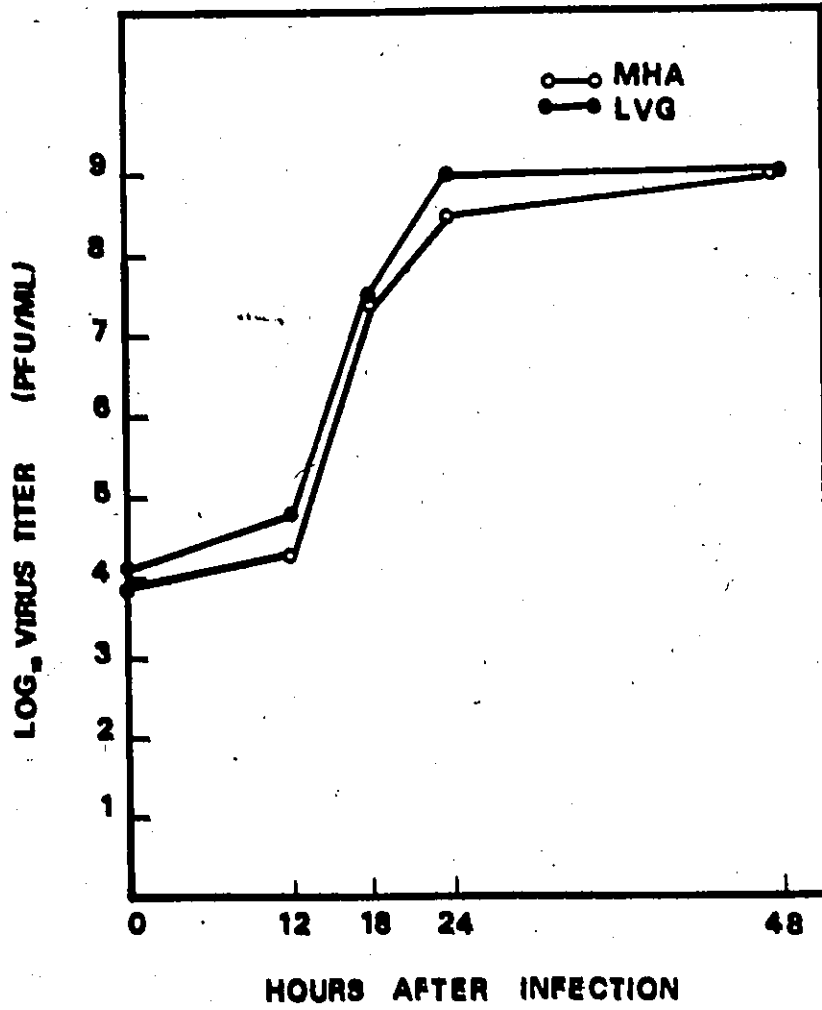
macrophages of both strains of hamsters supported virus
growth equally well.

4. <u>The Effect of Cyclophosphamide Immunosuppression
on Resistance to Infection</u>

The effect of cyclophosphamide immunosuppression
on virus-induced mortality was tested in both hamster
strains. For these experiments a single dose of cyclo-
phosphamide was administered 3 days after virus infection
and the mortality was compared in drug-treated and non-
treated animals which had been infected with Pichinde
virus. Figure 10 illustrates the results of a
representative experiment. Cyclophosphamide immuno-
suppression eliminated the age-acquired resistance of LVG
hamsters. The susceptibility of MHA hamsters to lethal
infection was increased slightly by cyclophosphamide
immunosuppression.

5. <u>Anti-Viral Antibodies in LVG and MHA Hamsters
after Pichinde Virus Infection</u>

Failure of animals to respond appropriately to
the antigenic stimulus of Pichinde virus during infection
may account for the lethality observed in MHA hamsters.
Antibody to the CF antigen of Pichinde virus was measured
at various intervals after infection in LVG and MHA
hamsters. Table VI shows CF antibody in these animals
at intervals after infection. No striking differences
existed in the level of CF antibody produced.

Figure 10

The effect of cyclophosphamide immuno-suppression on mortality in adult LVG and MHA hamsters. The animals were infected with 2.5×10^5 PFU of Pichinde virus i.p. then either given 100 mg/kg (MHA) or 150 mg/kg (LVG) of cyclophosphamide i.p. 3 days after infection (O—O) or left untreated (●—●). There was no mortality among hamsters given drug alone.
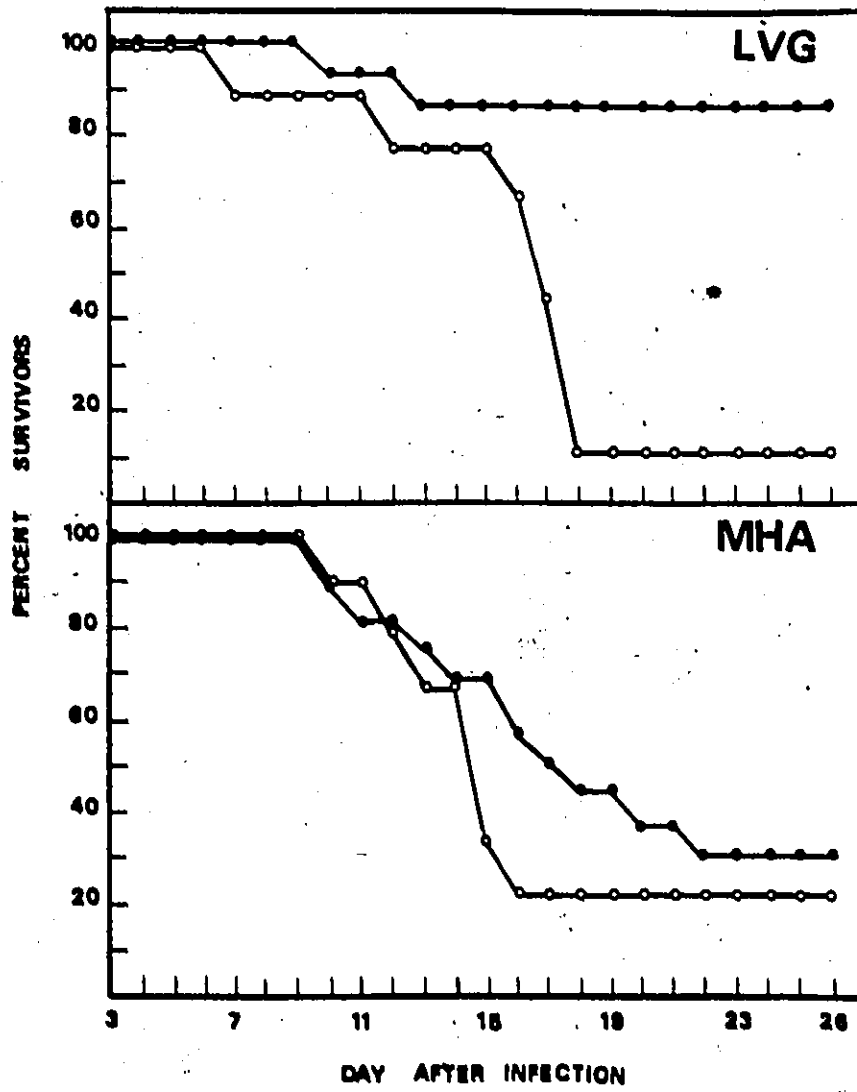
TABLE VI

MEAN COMPLEMENT FIXING ANTIBODY TITERS IN LVG AND MHA
HAMSTERS AT VARIOUS INTERVALS AFTER PICHINDE VIRUS
INFECTION

Hamster Strain	Day After Infection	CF Antibody Titer[1]	
		Virus Specific[2]	Anticomplementary[3]
LVG	10-13 (3)[4]	1:53[5]	$\leq$1:2
	21 (2)	1:256	$\leq$1:2
	30 (5)	1:128	$\leq$1:2
	60 (7)	1:64	$\leq$1:2
MHA	10-13 (5)	1:38	$\leq$1:2
	21 (2)	1:128	1:4
	30 (4)	1:128	1:21

[1] Highest dilution giving 50% endpoint in complement fix-
ation test against 5 units of antigen.

[2] Determined against antigen prepared from Pichinde virus
infected BHK_{21} cells.

[3] Fixation of complement in the absence of virus specific
antigen.

[4] Number of samples tested is indicated in parentheses.

[5] Geometric mean CF antibody titer.

6. Susceptibility of other Hamster Strains to
   Pichinde Virus Infection

Several other inbred hamster strains were
screened for susceptibility or resistance to Pichinde
virus infection. Animals of all of the strains tested
were given an identical dose of 2×10^5 PFU of virus
and then observed for mortality over a 25 day period.
Table VII summarizes the results of these experiments.
In the first experiment, only the MHA and CB strains of
hamster appeared to be susceptible, while the LVG,
LHC, PD-4 and LSH strains appeared resistant. In
a subsequent experiment LHC, PD-4 and CB hamsters were
infected with 2.5×10^5 PFU of virus and observed over
a 30 day period. Mortality was limited to 1 animal of
the LHC strain. MHA hamsters infected at the same
time and from the same virus pool, but for a different
experiment, became ill as previously observed. These
MHA animals are not shown in the table because they were
sacrificed for pathological study prior to actual death.
Total mortality observed for the two strains when data
from all experiments was pooled was 6% for LVG and 82%
for MHA hamsters.

7. Histopathology of Acute Pichinde Virus Infection
   in the Hamster

A preliminary histological study of organs re-
moved from Pichinde virus infected and control newborn

TABLE VII

MORTALITY AMONG VARIOUS HAMSTER STRAINS AFTER PICHINDE
VIRUS INFECTION

Hamster	Survivors[1]		Percent	
Strains	Initial Number		Mortality	
	Exp. 1	Exp. 2	Exp. 1	Exp. 2
LVG	20/23	-	13%	-
MHA	5/20	-	75%	-
LHC	6/6	10/11	0	9%
PD-4	6/6	5/5	0	0
CB	2/6	4/4	66%	0
LSH	3/3	-	0	-

[1] Survivors on the 25th day after infection.

LVG and adult LVG and MHA hamsters was carried out in collaboration with Dr. Frederick Murphy, Chief of the Viral Pathology Branch of the Center for Disease Control, Atlanta, Georgia. Kidneys, livers and brains were removed 8 days after infection from LVG hamsters infected as newborns and from age matched, non-infected hamsters. The same organs were also removed from virus-infected adult MHA and LVG hamsters on days 1, 8, 11 and 15 after infection with 2×10^5 PFU of virus intraperitoneally. All tissues were placed immediately into 10% buffered formalin and histologic sections were prepared and stained with hemotoxylin and eosin. Once prepared, the stained slides were sent to Dr. Murphy for histopathologic examination. A copy of Dr. Murphy's complete evaluation may be found in Appendix I. The important findings will be summarized below.

In newborn hamsters of the LVG strain, the predominate findings were extensive necrotic damage of the kidneys and liver and hemorrhagic lesions in the brain parenchyma. Severe necrosis of the liver was the main feature differentiating adult MHA from adult LVG hamsters. Kidney lesions in the adults of both strains were minimal compared to the lesions seen in newborn animals. Virus infected MHA adults showed a few necrotic foci within the brain parenchyma while adult LVG brains

appeared normal.

From these limited histologic studies, it appears that renal damage associated with brain and liver damage was responsible for death in the newborn hamsters. In the adult hamsters, the severity of the liver necrosis was sufficient to account for the deaths observed in MHA hamsters. A degree of hepatic involvement similar to that observed in MHA hamsters was not seen in the LVG hamsters.

C. Infection of Humans by Pichinde Virus

Human laboratory workers exposed to concentrated virus during the course of biochemical studies were shown by serological means to have been infected. Among persons working with Pichinde virus, 6 of 13 (46%) were found to have antibodies to the virus (Table VIII). No antibodies were found among 14 persons working in the same laboratory area where studies on the virus were being carried out but who were not working with the virus. Antibodies were not detected among any persons working in other areas of the same building.

Biochemical studies were performed by six workers, and in the course of these studies milligram quantities of purified virus were used. Antibodies were found among five of these six workers. Biological studies that entailed primarily preparation of virus stocks, virus

TABLE VIII

ANTIBODIES TO PICHINDE VIRUS AMONG LABORATORY PERSONNEL
WITH DIFFERENT EXPOSURES

Risk Group	No. Tested	CF Antibodies to Pichinde virus	
		No. Positive	% Positive
High	13	6	46
Moderate	14	0	0
Low	17[1]	0	0

[1] Paired serum samples on eight persons and single
serum samples on nine persons.

assays, and infection of animals were carried out by
seven workers, and only one of these persons developed
antibodies. This infection was putatively traced to
accidental self-inoculation that occurred while in-
jecting baby hamsters.

The antibody titers in relation to the time when
the persons began working with Pichinde virus are shown
in Figure 11. A fourfold rise in antibody titers is
evident with three persons, WR, FF and MB, and a four-
fold decline in titers is evident with two persons,
GH and BR. Serious illness was not associated with any
of the infections, although during the period under study
minor febrile illnesses were experienced. Because others
in the laboratory also experienced minor febrile illness,
it is not possible to attribute any specific illness
pattern to Pichinde virus infection.

In one case the period of infection was well
circumscribed. A worker (WR) concentrated Pichinde virus
on October 24, 1973, and no CF antibodies were found in
a serum sample taken October 30, 1973. Slight dysuria,
myalgia and pruritic vesicular eruption on the dorsum of
the left thumb developed on November 22, 1973. The
vesicles became hemorrhagic on the third day of the illness,
then resolved. Serum obtained November 28, 1973 had a
CF antibody titer of 1:16.

Figure 11

Complement fixing antibody titers to Pichinde
virus in laboratory workers in relation to the time
of possible exposure. The arrows indicate the time
when individuals began working with the virus.

CF ANTIBODY TITER (y-axis label)

MC — JUNE 1970
BR — JAN. 1971
GH — JULY 1971
FF
MB
WR

MARCH 1972 · JUNE 1972 · SEPT. 1972 · DEC. 1972 · MARCH 1973 · JUNE 1973 · SEPT. 1973 · DEC. 1973
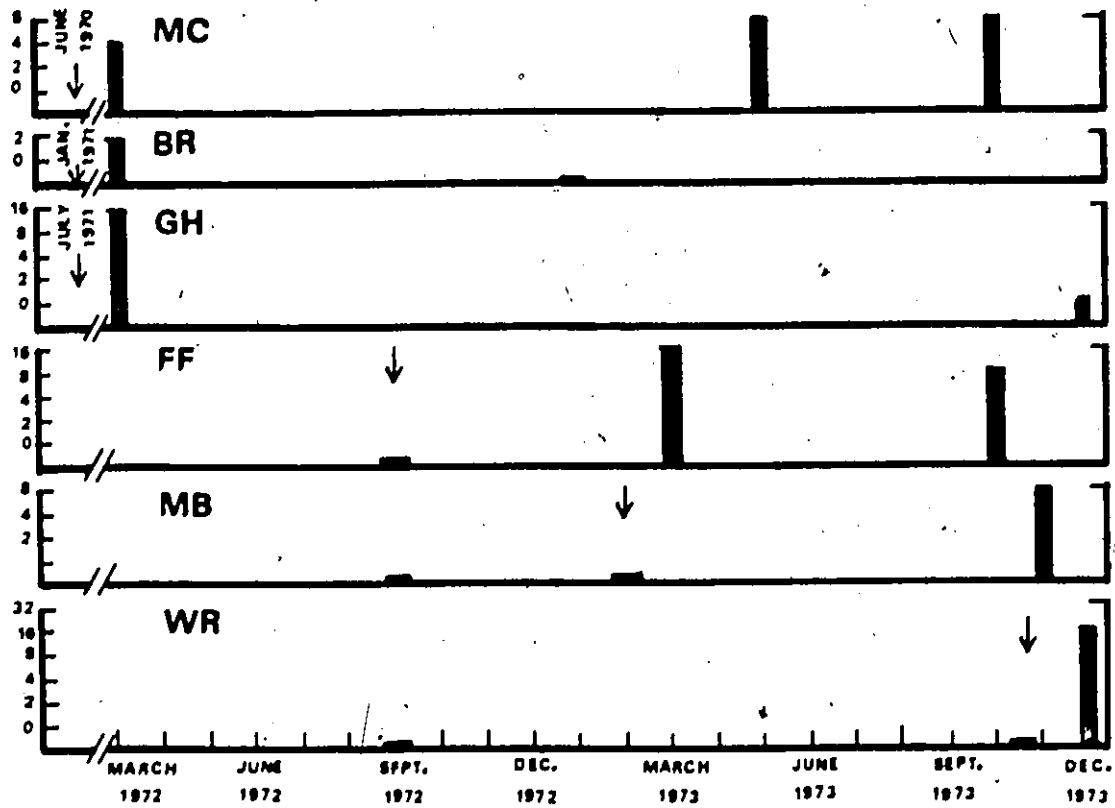
DATES OF BLEEDINGS

II. Antigen Synthesis and Expression in Cell Culture

Previous work (Carter, 1972) has established the kinetics of appearance of CF antigen in cell cultures infected by Pichinde virus. Cell associated CF antigen first appears at 8-12 hr after infection and increases in concentration to a maximum level at 72-96 hours. Antigen released into the supernatant does not reach high titers over this time period, and for this reason, only cells harvested 72 hours (and occasionally 96 hours) after infection were utilized as a source of CF antigen. This practice was economical, since virus concentrations reached maximum levels at 72 hours after infection, thus both the cells and their supernatant fluids could be utilized.

A. Virus-specific Antigen Expression Correlated with New Virus Production in Infected Vero Cells

One potential mechanism by which persistent viruses may escape the host defenses is by presenting a minimal amount of antigen on the cell surface. This would serve not only to minimize the antigenic stimulus, but would also present a limited target for the antibody or cell-mediated immune effector systems. To test the possibility that antigen expression is regulated or modulated in Pichinde virus-infected cells, an experiment was performed to measure the relationship between virus

production, surface antigen expression, and intracellular CF antigen levels in infected Vero cells. Identical coverglass cultures were seeded in shell vials and after 24 hr the cells were infected with Pichinde virus at an MOI of 1-3 PFU/cell. Following infection the coverslips were overlaid with maintenance medium and incubated. The medium was changed at 24 hr intervals on all coverslips. At time intervals of 1, 6, 12, 24, 48, 72, 96 and 120 hours after infection, virus-infected and control coverslips were removed and both surface and cytoplasmic immunofluorescent staining were done. Samples of culture medium were removed and reserved for virus titration to determine new virus production over the previous 24 hr interval. The results of this experiment are shown in Figure 12. New virus production and the percentage of cells expressing surface antigen were essentially parallel; both increased after 12 hr, peaked at 24-48 hr, then declined sharply at later sampling times. The percentage of cells expressing cytoplasmic antigen peaked at 46% at 24 hours and remained relatively constant thereafter. With daily medium changes the cells showed no cytopathogenic effect which could be detected in viable preparations or in Giemsa stained specimens. The total numbers of

Figure 12

      Expression of cytoplasmic and surface
antigen in relation to infectious virus production
after Pichinde virus infection in Vero cells. Vero
cell cultures on coverslips were infected with Pichinde
virus (MOI 1) and incubated. At the sampling times
indicated, 2 coverslips were stained for surface
antigen and 2 stained for cytoplasmic antigen. The
supernatant fluid from these coverslips was assayed
for infectious virus. Medium was changed on all
cultures at 24 hour intervals; virus titers represented
virus production over the previous 24 hours.
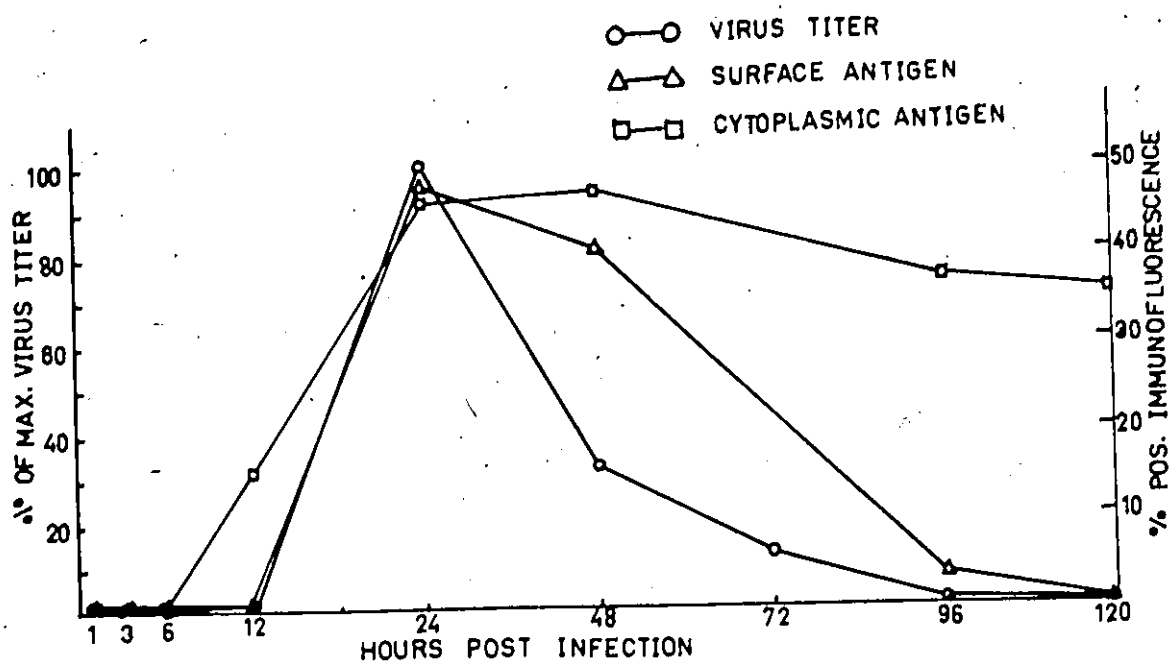
cells present on the coverslips after 120 hours were similar for both infected and control cultures.

B. Virus Replication and Antigen Production in Cells Treated with Actinomycin D

Actinomycin D has been shown to inhibit the replication of arenaviruses (see Introduction). The point of this inhibition in the replicative cycle of the virus has not been determined beyond an apparent dependence on host transcription. Experiments designed to determine with greater accuracy the point of inhibition of replication in the presence of actinomycin D were performed. The synthesis of CF and surface antigens was quantitated and correlated with virus replication in cell cultures. Identical confluent monolayers of BHK_{21} cells were infected with Pichinde virus at a multiplicity of infection of 1-3 PFU/cell. Following adsorption, maintenance medium containing 0, 1, 2 or 3 ug/ml actinomycin D was added to each flask. After 48 hours of incubation supernatant fluids were removed from each flask and assayed for infectious virus. The cell monolayers were scraped off the flasks with a rubber policeman and washed with PBS. The cells were then resuspended in 2 ml of PBS and lysed by two cycles of freeze thaw followed by sonication. Protein

content of the cell lysates was determined, and the CF antigen content was measured.

A ratio of CF antigen (CF units/mg protein) was obtained for each of the drug treated and control cultures. Virus titers in the supernatant fluid were expressed as the percent of control virus levels and compared with the CF units/mg protein as shown in Table IX. Virus titers were decreased by approximately 99% in the presence of 1-3 ug/ml actinomycin D. In contrast, CF antigen concentration was decreased by only 35% at 1 ug/ml of drug and remained at that level with increasing drug dosage. Immunodiffusion analysis of the drug treated cells revealed that both of the antigens detectable by this method were present in the drug treated cells (Figure 13).

Cells incubated in the presence of AD were examined by direct immunofluorescent staining in order to quantitate the percentage of infected cells in the cultures. In the absence of the drug, essentially 100% of the cells stained for Pichinde virus-specific antigen in the cytoplasm 48 hours after infection. In the presence of drug at concentrations of 1 and 4 ug/ml 66% and 64% of the cells expressed viral antigen in the cytoplasm. The decrease observed in antigen content

TABLE IX

PICHINDE VIRUS PRODUCTION AND ANTIGEN SYNTHESIS IN BHK$_{21}$ CELLS INFECTED AND
CULTURED IN THE PRESENCE OF VARIOUS CONCENTRATIONS OF ACTINOMYCIN D

Actinomycin D Concentration ug/ml	Virus Titer[1]	Percentage of Maximum Virus Yield	Cell Associated CF Antigen Units/mg Protein[2]	Percentage of Cells Infected[3]
0	8×10^7	100	7	100
1	4×10^6	5	4.9	70
2	-	-	4.6	66
3	4×10^5	0.5	4.5	64

1 Virus titer in supernatant fluids measured 48 hr after infection.

2 Cell associated antigen detected in cell lysates by complement fixation tested 48 hr after infection.

3 Percentage of cells infected determined by direct immunofluorescent staining of acetone fixed cells.

Figure 13

Immunodiffusion test showing antigens
produced in BHK_{21} cells incubated with and without
actinomycin D. The antigens used were: A. high
titered soluble antigen included as a positive
control; B. lysate of infected cells incubated with
2 ug/ml of actinomycin D; D. lysate of cells not
treated with drug; C. purified Pichinde virus
($\sim$ 500 ug/ml viral protein) disrupted by treatment
with 1% NP-40 and RNase; S. Pichinde virus antiserum.
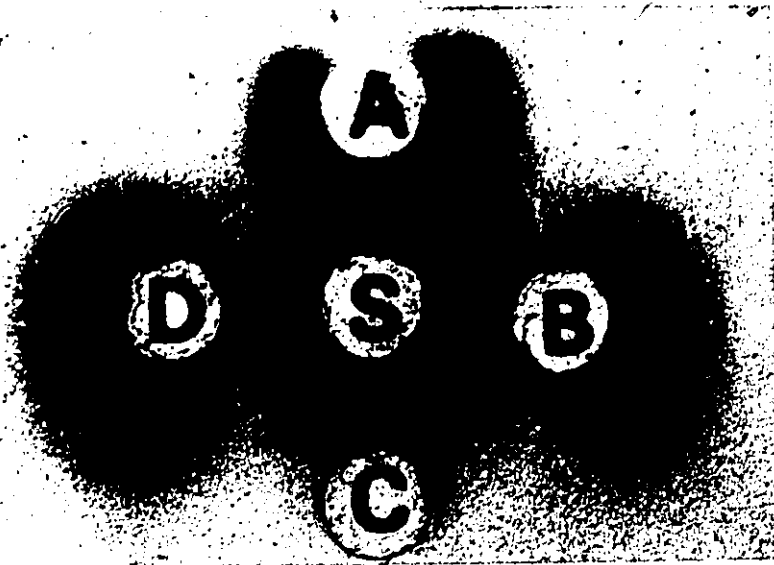
reflects the decrease in cells infected in the cultures.
Morphologically, the cytoplasmic antigen in AD treated
cells and control cells was indistinguishable as
assessed by immunofluorescent staining.

Immunofluorescent staining of the surface of
virus infected cells cultured in the presence and
absence of AD showed significant differences in the
pattern and intensity of the staining. Cells infected
by Pichinde virus and treated with AD stained more
intensely at the surface than infected cells not treated
with the drug. The pattern of staining of the cyto-
plasmic membrane was different for the two cell types
(Figure 14). Diffuse staining uniformly covered the
membrane of the drug treated cells (Fig. 14a). Non-treated cells
stained in a "patchy" or beaded manner (Figure 14b).

To quantitate the expression of surface antigen
on cells incubated in the presence and absence of AD,
an antibody binding assay was performed. IgG from
hamsters immune to Pichinde virus and normal hamster IgG
were purified and radiolabelled with ^{125}I. Aliquots of
the labelled immune and normal IgG were reacted as described
in Materials and Methods with infected cells which had
or had not been incubated with AD (2 µg/ml) and harvested
at various intervals after infection. The results of one

Figure 14

Immunofluorescent staining of the surface
of cells infected with Pichinde virus. BHK_{21} cells
were infected with Pichinde virus, incubated 24 hr.
at $37^{o}C$, trypsinized and stained with FITC conjugated
IgG from immune hamsters. Top, cells incubated in
the presence of 1 ug/ml of actinomycin D; bottom,
no actinomycin D.
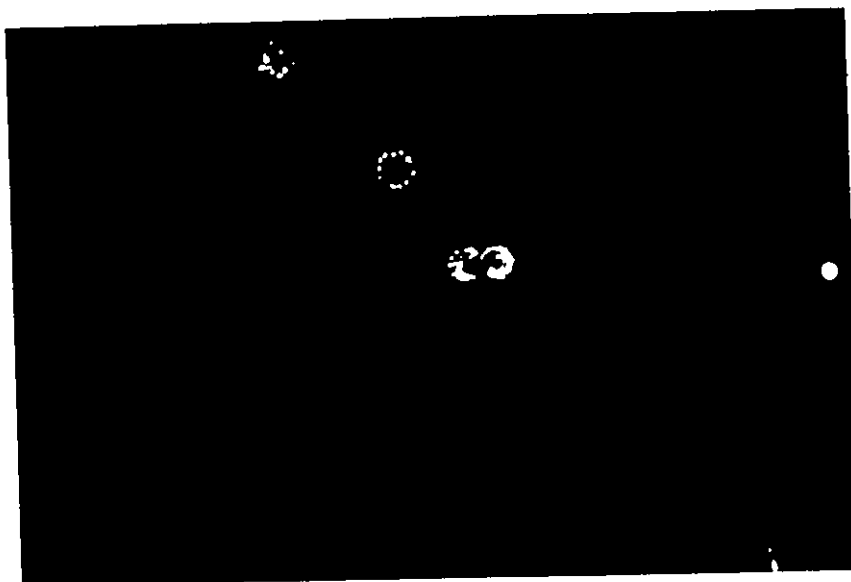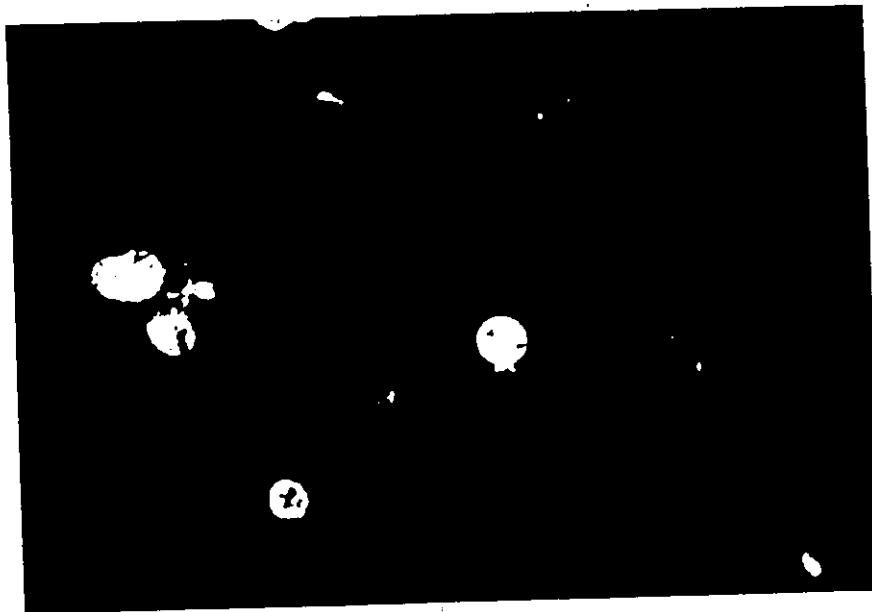
such antibody binding assay are shown in Figure 15.

From these data it appeared that virus specific antigens

are present on the surface of cells as early as 6-12

hours after infection. Surface antigen appeared to

accumulate at a similar rate in both drug-treated and

non-treated cells for the first 24 hours; by 48 hours

after infection the AD treated cells bound significantly

more IgG. These results suggest that accumulation of

virus antigen occurs on drug-treated cells.

C. Polypeptide Composition of Cells Incubated in

the Presence and Absence of AD

In a single experiment, the polypeptide composition

of infected and control cells grown in the presence and

absence of actinomycin D was examined. Six identical

cultures of BHK_{21} cells were grown in 75 cm^2 culture

flasks. Four of these cultures were infected with Pichinde

virus, 2 at a MOI of 1 PFU/cell and 2 at 10 PFU/cell.

The remaining 2 flasks were mock infected. After

adsorption, one of each of the 3 pairs of flasks was

overlaid with MEM containing 2 μg/ml actinomycin D. The

other flasks were overlaid with MEM without drug. Six

hours after infection 5 μCi/ml ^{3}H-L amino acid mixture

was added to all of the flasks. After 24 hours of

incubation all of the flasks were drained and washed with

PBS. The washed cells were then solubilized and prepared

Figure 15

Quantitation of surface antigens in act-
inomycin D treated cells using ^{125}I labelled antibody.
BHK$_{21}$ cells were infected with 1-3 PFU/cell and
incubated in the presence or absence of 1 ug/ml of
actinomycin D. At intervals the cells were mono-
dispersed and reacted with anti-Pichinde virus IgG
which had been labelled with ^{125}I. Infected cells,
no actinomycin D (△--△); infected cells and
actinomycin D (O—O). Uninfected cells, no
actinomycin D (▲--▲); uninfected cells and act-
inomycin D (●--●).
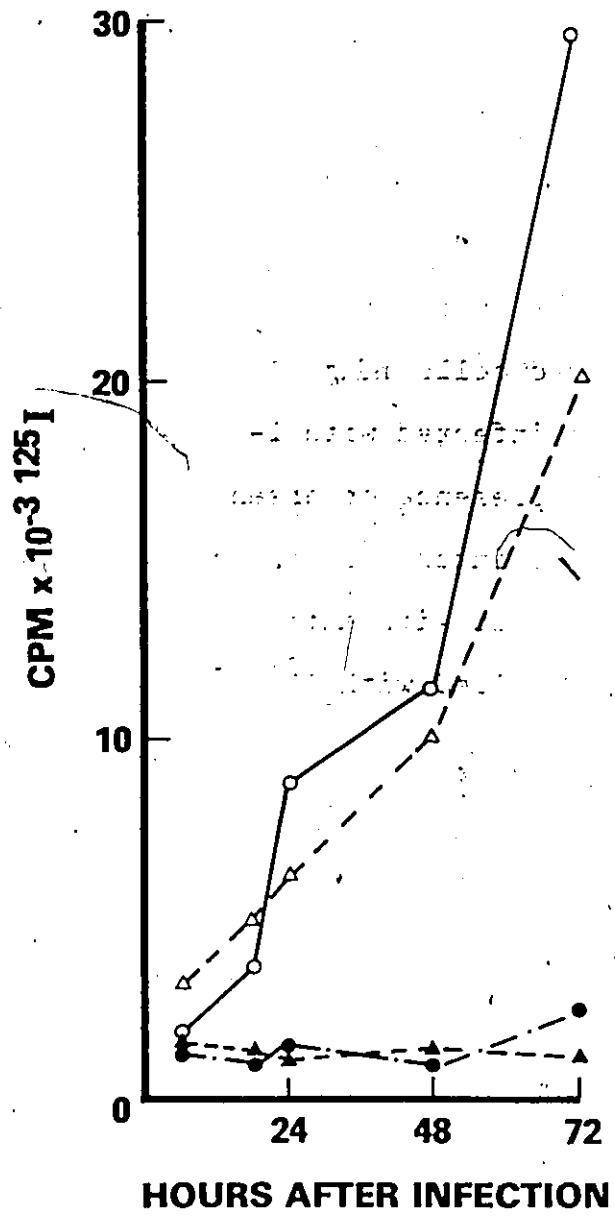
for SDS-PAGE. A slab gel was run of the samples with glucosamine labelled and amino acid labelled virus marker samples.

The autoradiogram of this gel is shown in Figure 16. A polypeptide comigrating with the major viral polypeptide, V_I, was found in the infected cells both in the presence and absence of drug. This polypeptide was not found in non-infected cells. None of the other viral polypeptides appeared by this method to be unique to the infected cells.

From the results of these experiments it appears that virus-specific protein synthesis occurred in BHK_{21} cells infected with Pichinde virus and incubated in the presence of AD. Two antigens detectable by immunodiffusion as well as virus specific surface antigens were demonstrable in these cells. Other experiments (data not shown) showed that the decrease in infectious virus production was not accounted for by an increase in cell-associated virus. Both cell-associated and fluid-associated virus titers were decreased to a similar extent by AD.

The possibility existed that the decreased virus titer in AD-treated cells represented an increased production of defective interferring (DI) particles. To examine this, supernatant fluids from AD-treated cultures

Figure 16

Virus-specific polypeptides present in BHK_{21} cells infected with Pichinde virus and incubated with or without actinomycin D. Six 75 cm^2 flasks of BHK_{21} cells were treated as follows: Two of the flasks were mock infected, two were infected at a MOI of 1 PFU/cell and two more infected with 10 PFU/cell. After adsorption the monolayers were washed and maintenance medium with 2 ug/ml actinomycin D was added to one flask of each type. To the other flask, maintenance medium without drug was added. Six hours after infection 5 uCi/ml ^{3}H-L amino acid mixture was added to each flask, and incubation was continued. At 24 hours after infection, all of the monolayers were washed with PBS then solubilized in 1% SDS, 1% 2-mercaptoethanol, 10% glycerol and prepared for slab gel electrophoresis. 100 ul of the samples were electrophoresed on a 10% poly-acrylamide gel, and the radiolabelled polypeptides were detected by fluoroautoradiography. The lanes contained the following (1)-^{3}H glucosamine labelled Pichinde virus marker (80,000 CPM). (2 and 9) ^{3}H amino acid labelled virus marker (100,000 CPM). (3) cells infected at MOI = 1 and incubated without drug, (4) cells infected at MOI = 1 and incubated with drug. (5) cells infected at MOI = 10, no drug, (6) cells incubated at MOI = 10, with drug. Control-non infected cells incubated without and with actinomycin D were run in lanes (7) and (8) respectively.

V_I
V_II

V_III

V_IV

V_I
V_II

V_III

V_IV

1 2 3 4 5 6 7 8 9

radiolabelled with ^{3}H-amino acids were subjected to the standard virus purification used for Pichinde virus. Fractions from the final 20-50% w/w sucrose gradient were harvested and assayed for radioactivity and for infectivity. The results presented in Figure 17 demonstrate that there was a proportionate decrease in both ^{3}H-amino acid content and infectivity in the fluids from AD treated cultures. No evidence was found to support the concept of increased DI particle production.

Figure 17

Decreased particle production in cells
infected with Pichinde virus and incubated in the
presence of actinomycin D. BHK_{21} cell monolayers
in two 75 cm^2 flasks were infected with 1-3 PFU/cell
of Pichinde virus.   After adsorption 1 ug/ml of
actinomycin D was added to one flask while the other
received no drug. Two uninfected flasks were treated
similarly. To all flasks, 0.5 uCi/ml of ^{3}H-amino
acid mixture was added. The fluids were harvested after
24 hr at 37°C, precipitated with PEG, resuspended in
TNE and centrifuged through 20% sucrose onto a 50%
sucrose cushion. The material at the interface was
aspirated by side puncture and centrifuged on a con-
tinuous gradient. Fractions from the gradient were
assessed for radioactivity and for infectious virus.
The radioactivity plotted in the figure represents the
CPM in the fraction of the material from the infected
cultures minus the CPM of the corresponding fraction of
the material from the control cultures. Infectious virus,
no actinomycin D (△--△); infectious virus, actinomycin
D (▲...▲); ^{3}H-amino acids, no actinomycin D (O--O);
^{3}H-amino acids, actinomycin D (O—O).
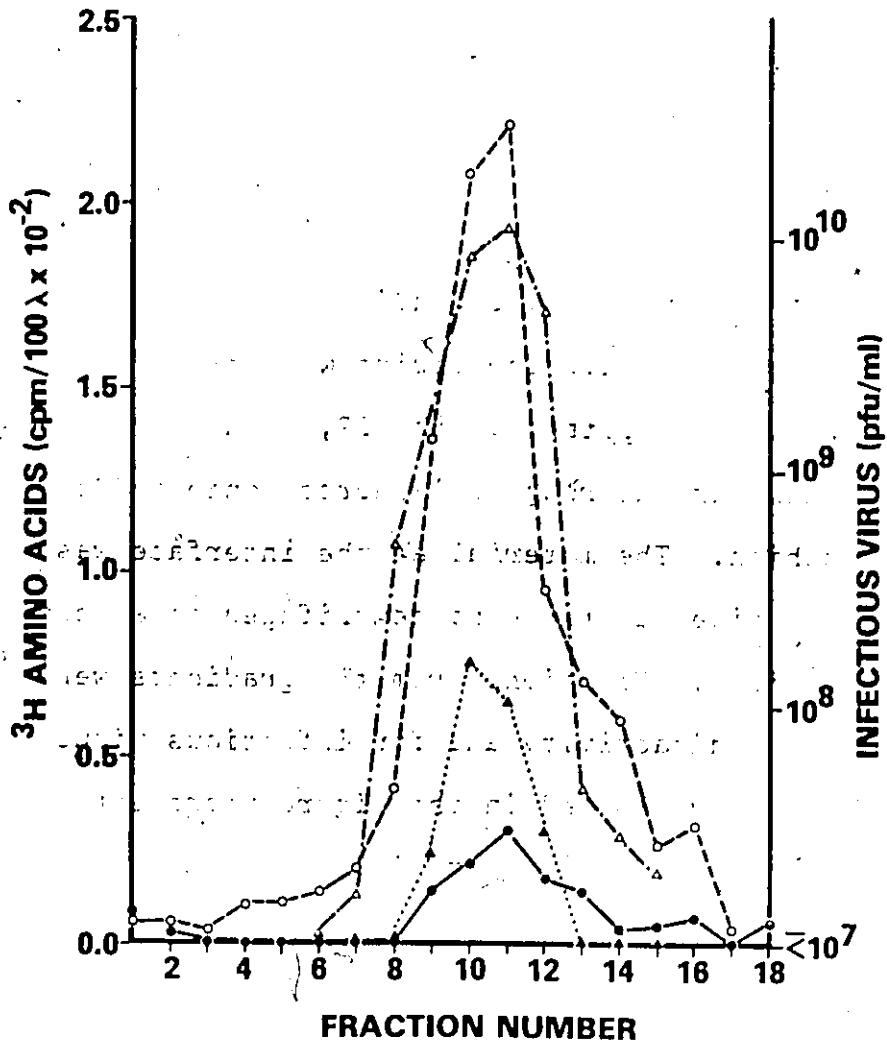
III. Biochemical Studies of the Antigens of Pichinde
     Virus

A. Soluble Antigens Detectable in Lysates of Cells
   Infected by Pichinde Virus

Two virus-specific antigens were detectable by
immunodiffusion in soluble extracts of BHK_{21} cells
infected by Pichinde virus. The two antigens were found
to differ in their thermal lability as well as their
resistance to the proteolytic action of pronase.
Figure 18 demonstrates the differential susceptibility
of the antigens upon heating at $56^{\circ}C$. One antigen was
relatively stable at $56^{\circ}C$ while the other antigen could
no longer be detected by immunodiffusion after 20 minutes
at $56^{\circ}C$. When soluble lysates were exposed to 25 ug/ml
of pronase for 60 min at $37^{\circ}C$, the heat stable antigen
was also found to resist proteolytic digestion while
the heat labile antigen was no longer detectable after
proteolytic digestion. Table X summarizes the effect of heat
and   enzymatic digestion on the antigens. The antigen
unaffected by heat and pronase digestion was called the
major antigen while the heat labile, pronase sensitive
antigen was called the minor antigen. Neither antigen
was affected by digestion with 25 ug/ml of ribonuclease-a
for 60 min at $37^{\circ}C$. By autoradiography of dried immuno-

Figure 18

Immunodiffusion test showing the effect of heating on the antigens detected in lysates of BHK_{21} cells infected with Pichinde virus. The perimeter wells contained soluble antigen which was heated at $56^{o}C$ for 1 min (1), .5 min (2), 10 min (3), 20 min (4), 30 min (5) and 60 min (6). The center wells contained immune guinea pig serum undiluted (left) or diluted 1:2 (right).
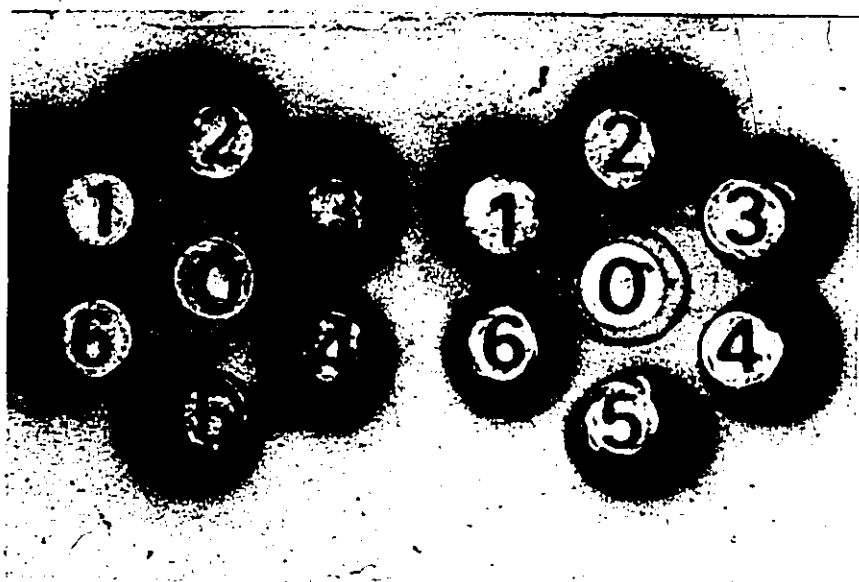
TABLE X

SUMMARY OF THE EFFECT OF HEAT AND ENZYMATIC DIGESTION ON

PICHINDE VIRUS ANTIGENS EXTRACTED FROM INFECTED BHK$_{21}$ CELLS

Treatment	CF Titer[1]	Immunodiffusion Band Observed	
		Major	Minor
None	128	+[2]	+
Incubation at 22°C 24 hr	128	+	+
Incubation at 56°C 60 min	86	+	−
Digestion with 25 ug/ml RNase at 37°C 60 min (pH 7.2)	128	+	+
Digestion with 25 ug/ml pronase at 37°C 60 min (pH 7.2)	ND	+	−

[1] Reciprocol of antigen dilution yielding 50% end-point of complement-fixation test. Mean value of 3 determination.

[2] + Indicates presence of the precipitin band.

[3] ND – Not Done.

diffusion slides, both antigens were shown to be labelled with ^{14}C arginine.

B. Some Biophysical Properties of the Major Antigen

A lysate of cells infected with Pichinde virus was centrifuged at 100,000 x g for 60 min then applied to a G-200 Sephadex column which was previously calibrated with a series of proteins of known molecular weight. The antigenic activity, as assessed by CF, eluted from the column in a peak at a Ve/Vo value of 2.0-2.1. When the location of the antigen peak was plotted with the marker proteins (Figure 19), this value corresponded to a molecular weight of approximately 2×10^{4}. The identity of the antigen peak as the major antigen was confirmed by immunodiffusion. The minor antigen was not recovered as a distinct peak following gel filtration.

The sedimentation rate of the antigenic activity was determined in 5-20% W/V sucrose gradient by the method of Martin and Ames (1961). The position of peak of activity relative to 7S and 4.4S marker proteins was found to be 3.5S. The major peak of CF activity was shown by immunodiffusion to contain both the major and the minor antigens. The minor antigen detected by immunodiffusion was found predominately in the faster sedimenting half of the antigen-containing peak, and overlapped the slower sedimenting major antigen.

Figure 19.

        Estimation of the molecular weight of the
CF antigen of Pichinde virus by gel filtration in
G-200 Sephadex. A 1.5 X 55 cm column was equilibrated
with BBS, pH 8, (1:4000 NaN$_3$) and standardized using
the indicated marker proteins. A lysate of virus-
infected BHK$_{21}$ cells was clarified by centrifugation
at 100,000 x g for 60 min, and 1.2 ml of the super-
natant was applied to the column. Fractions of 2 ml
were collected and assayed for antigen by CF.

FRACTION NUMBER

60

50 — RIBOFLAVIN

40 — CYTOCHROME C 12,400
ANTIGEN PEAK (3 RUNS)

MYOGLOBIN 17,800

30 — HEMOGLOBIN 65,000
PHOSPHORYLASE A 94,000
HUMAN IgG 160,000

20 — BLUE DEXTRAN

10

2   4 6 8 10   2   4 6 8 10   2   4 6 8 10   2   4 6 8 10   2
×10² ×10³ ×10⁴ ×10⁵

MOLECULAR WEIGHT

ELUTION VOLUME / VOID VOLUME

3.0

2.0

1.0
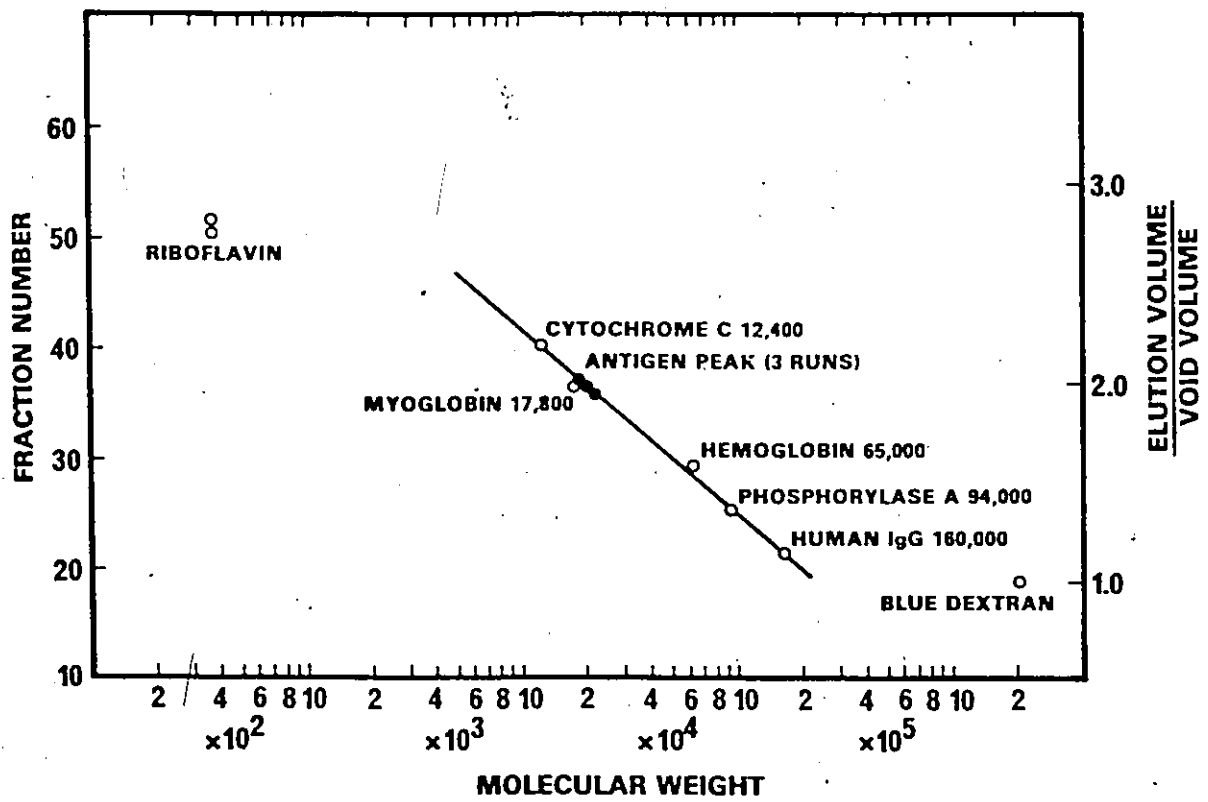
Antigen which had been heated to 56°C for 60 min showed
a marked decrease in activity in the sedimenting peak,
but no change was observed in the position of the major
peak of CF activity (Figure 20).

Isoelectric focusing in a preparative column
was carried out using soluble antigen prepared from
infected cell lysates. The profile of CF activity
recovered in a typical run using a pH 3.5-10 gradient
is shown in Figure 21a. A peak of antigenic activity
focused at pH 5.2 with a variable shoulder at higher
pH values. The identity of the pH 5.2 antigen peak was
shown by immunodiffusion to be the major antigen. Figure
21b shows the pattern of radiolabelled protein obtained
when a parallel control cell lysate was focused on an
identical pH 3.5-10 gradient. The distribution of
radiolabelled proteins obtained from infected cells is
similar if not identical to that obtained from control
cells. The soluble antigen would appear to comprise a
relatively minor proportion of the total soluble protein
of the infected cells.

C. Purification of the Major CF Antigen from Infected
   BHK_{21} Cells

Taking advantage of the characteristics described
above, a scheme was derived to partially purify the major
CF antigen from lysates of infected BHK_{21} cells. The methods

Figure 20

Sedimentation of soluble antigen in a
5-20% sucrose density gradient. A soluble extract of
virus-infected cells was prepared and either heated
to 56°C for 60 min (△─△) or used without further
treatment (●─●). The sample was centrifuged
for 22 hr at 35,000 RPM (Spinco SW-41 rotor). The
antigenicity in collected fractions was determined
by CF. The positions of marker proteins, human IgG
(7S) and hemoglobin (4.4S), run on a separate
gradient, are shown.
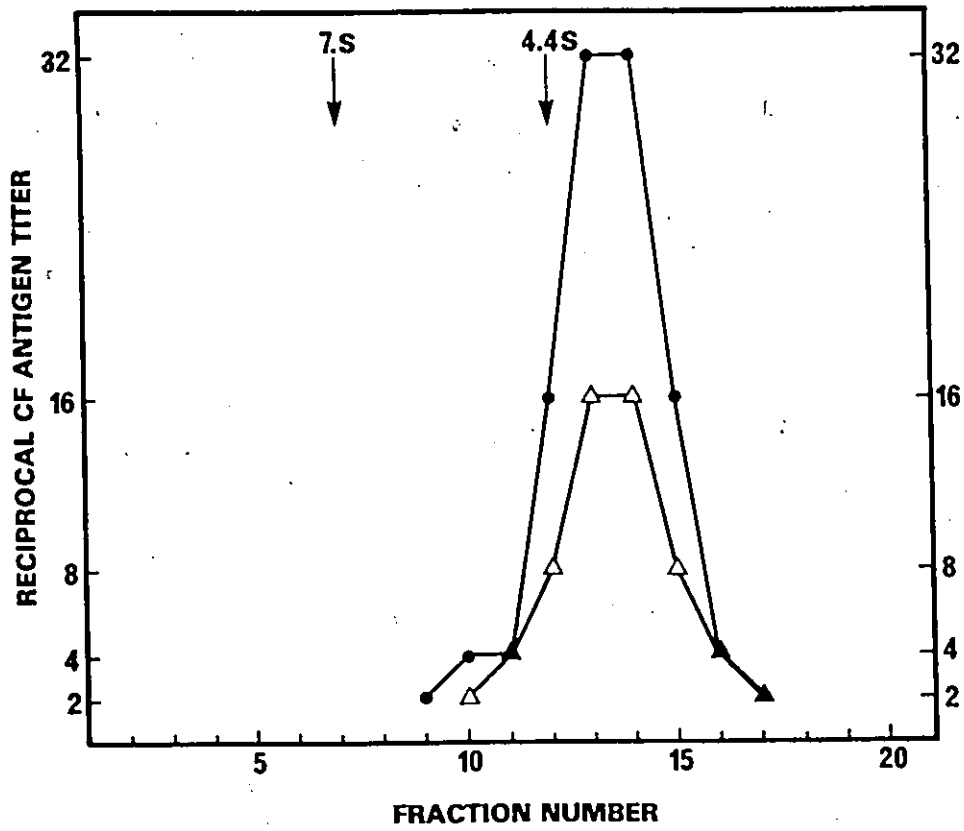
Figure 21

     Isoelectric focusing of cell-derived
Pichinde virus-specific and control antigens.
Soluble lysates of infected (TOP) and control
(BOTTOM) BHK_{21} cells were first centrifuged on
preparative 5-20% sucrose gradients, then dialysed
against 1% glycine overnight. The dialysed samples
were incorporated into identical pH 3.5-10 iso-
electric focusing gradients and focused for 72 hr
at 300 v. Fractions were collected and the radio-
activity and CF antigen activity determined in each.
(●—●)radioactivity; (○—○) CF antigen; and
(- - - -) pH.
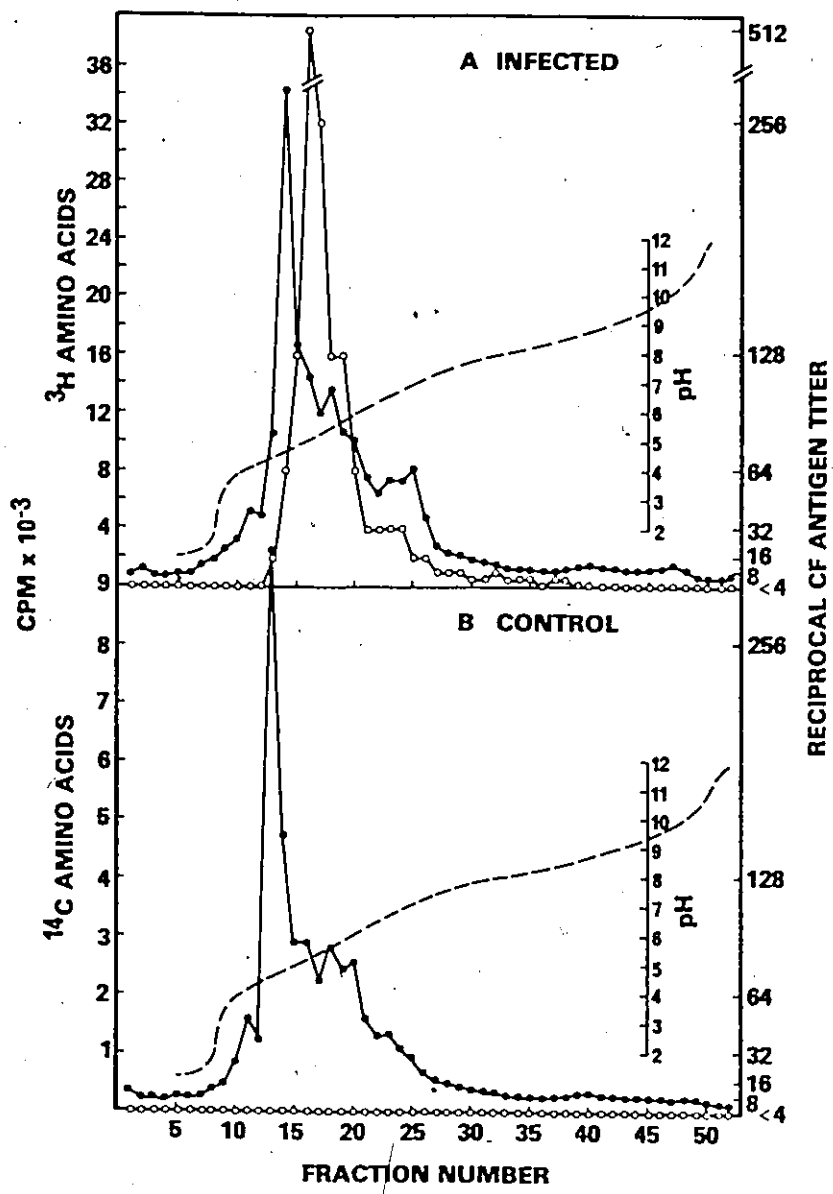
used and the recovery of antigen at each step are
shown in Table XI. The increase in CF antigen units
is indicative of the degree of purification obtained.
The Purification index of the Biogel P-100 antigen pool
was 20.

An experiment performed since the data in
Table XI was obtained has demonstrated that Biogel P-200
gel filtration of the antigen pool obtained by isoelectric
focusing yielded a single isolated peak of antigenic
activity coincident with a peak of ^{3}H amino acid label.
Parallel preparations from non-infected cells yielded no
peak of radiolabel eluting in the same region (Figure 22).
The approximate molecular weight of the eluting antigen-
icity relative to protein markers was 28,000.

Antigen which was partially purified by differential
centrifugation, 5-20% sucrose gradient centrifugation
and isoelectric focusing was used to prepare antisera in
guinea pigs. The sera of 2 immunized animals had CF
titers of 1:64 against the virus-induced antigen. A
pool of these sera gave a band which was identical to
the major antigen by immunodiffusion. By indirect
immunofluorescence using BHK_{21} or Vero cells infected
with Pichinde virus and the guinea pig antiserum to the
major antigen at a dilution of 1/16, a granular cytoplasmic

TABLE XI

SUMMARY OF PURIFICATION OF CF ANTIGEN FROM BHK$_{21}$ CELLS INFECTED WITH PICHINDE VIRUS

Stage of Purification	Total Prot. Recovered (mg)[1]	Total Antigen Recovered[2] (CF Units)	Specific Activity CFU/mg Prot.[3]	Purification Index[4]
1. Crude 20% v/v cell suspension	>100	>20,000	ND	ND
2. 12,000 x/g supernatant	50	15,000	300	1
3. 100,000 x g supernatant	24.5	14,745	602	2
4. 5-20% sucrose gradient antigen pool	11.0	14,080	1280	4.2
5. Isoelectric focusing antigen peak (pH 5.2)	3.15	6,400	2050	6.8
6. Biogel P-100 antigen pool	0.51	3,060	6000	20

1 Protein content determined by the method of Lowry et al (1951).

2 CH$_{50}$ antigen units in test with 4 units of immune hamster serum.

3 $\dfrac{\text{Total CF antigen units}}{\text{Total mg protein}}$

4 $\dfrac{\text{Specific activity of 12,000 x g supernatant}}{\text{Specific activity at subsequent stage of purification}}$ X 10^{-1}

Figure 22

Biogel P-200 gel filtration of Pichinde
virus-specific CF antigen. Soluble lysates derived from
virus-infected BHK_{21} cells grown in the presence of
5 uCi/ml ^{3}H-amino acids and from control cells grown
in the presence of 0.5 uCi/ml ^{14}C amino acids were
partially purified by rate zonal sedimentation and
isoelectric focusing. The fractions containing the major
peak of CF antigen and the corresponding fractions from
the control were dialysed against BBS, pH 8, then 4 ml
of the dialysed sample was applied to a 2.5 X 90 CM
Biogel P-200 column equilibrated with BBS. In two
separate runs the antigen-containing and control pre-
parations were eluted and the radioactivity and CF
antigen in each fraction was determined. The symbols
are: (●—●) CPM ^{3}H-amino acids in material containing
antigen; (△—△) CPM ^{14}C amino acids in the control
preparation, and (O--O) CF antigen activity. No
CF antigen activity was detectable in the control
preparation.
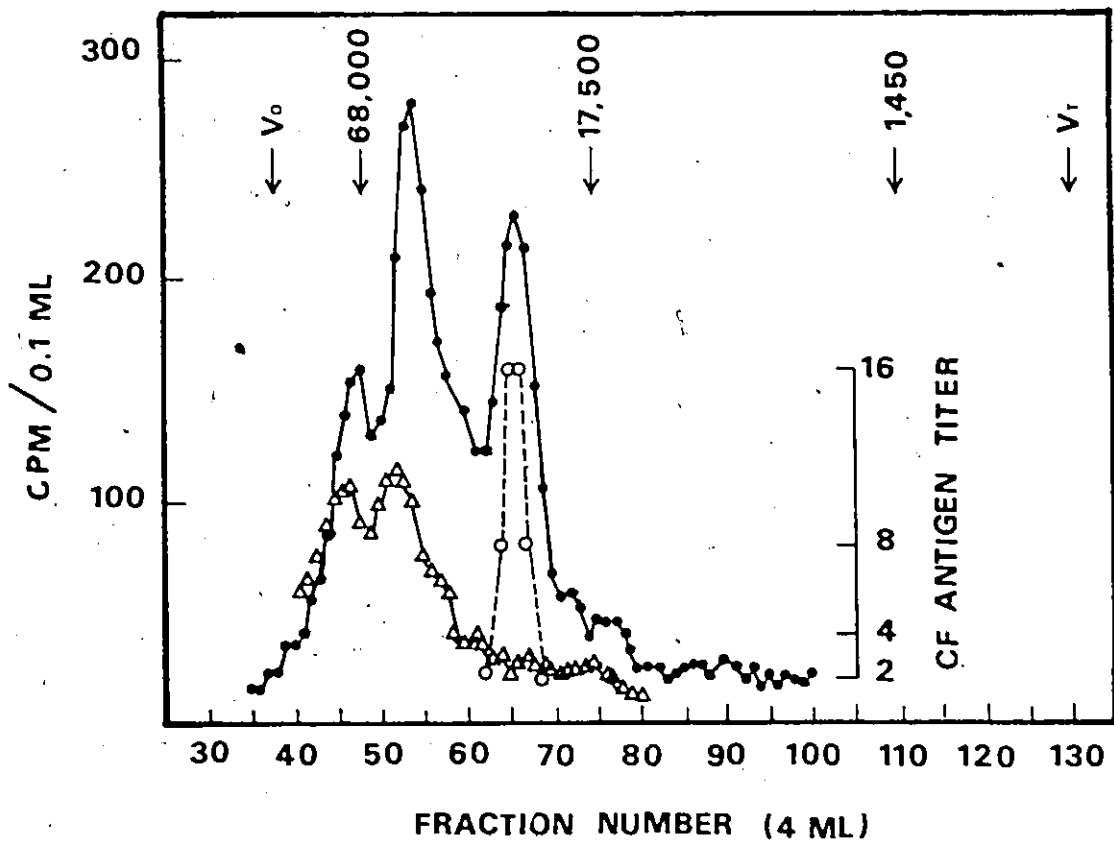
staining was observed in acetone fixed cells (Figure

23a). Corresponding control cells gave no reaction at

a comparable serum dilution (Figure 23b). The staining

pattern observed with antisera to the partially

purified antigen was similar to that observed when the

cells were reacted with immune sera from guinea pigs or

hamsters (Figure 24).

Indirect immunofluorescent staining of the

surface of viable cells was performed using sera from

hamsters or guinea pigs infected with the virus and

antiserum against partially purified major antigen. Virus-

specific staining of the surface of the infected

cells was obtained with immune sera from both hamsters

or guinea pigs, but not with antiserum to the major

antigen. These data suggest that the major antigen is

not expressed on the surface of cells infected with

Pichinde virus.

D. Relationship of the Major Antigen with Structural

Components of the Virus

The relationship of the antigen derived from

infected cells with the known structural components of

the virion was first approached by disrupting purified

virus with detergent treatment, separating the virion

components by centrifugation and immunizing guinea pigs
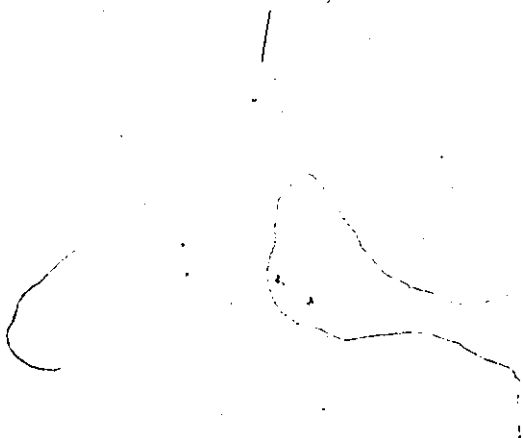
Figure 23

     Indirect immunofluorescent stain of Pichinde
virus infected Vero cells by antiserum against
partially purified, heat stable CF antigen. Top,
infected cells; bottom, control cells.

Figure 24

        Direct immunofluorescent stain of Vero
cells infected with Pichinde virus using immune
hamster globulin conjugated with FITC.

with the various viral fractions. The scheme is shown
in Figure 25. Purified Pichinde virus was disrupted with
0.2% NP-40, and the treated preparation was centrifuged
to remove the insoluble components, or "cores", from
the solubilized components. The separated components
were used to immunize guinea pigs as described in
Materials and Methods. The resulting antisera were tested
for antibody activity by CF, immunodiffusion, and in-
direct immunofluorescence. Early sera from animals
receiving virus "cores" yielded, by immunodiffusion,
one band when reacted with lysates of infected cells.
This band identified with the band detected by antisera
prepared against purified major antigen and with the
major antigen detected by immune serum (Figure 26).
The material solubilized from purified virus failed to
induce a significant antibody response in the immunized
animals.

The reactivities of the antisera against virion
"cores" and soluble material were assessed by indirect
immunofluorescence. Staining of cytoplasmic antigen
was observed with antisera directed against intact
virus, virion "cores" and solubilized materials. With
the antisera against intact virus and against virion cores
the reactions were similar and consisted of a granular

Figure 25

    Flow chart of the methods used to obtain
antisera directed against components of NP-40
disrupted Pichinde virus.

PURIFIED PICHINDE VIRUS

NO TREATMENT   NP-40 ADDED TO 0.2%

                                INCUBATE 37°C 45min

                                CENTRIFUGE 150,000 g
                                        90min

3.5 x 10^{10} PFU/ml   SUPERNATANT   PELLET 0 PFU/ml
                             0 PFU/ml

CFA   CFA   CFA

GUINEA PIGS   GUINEA PIGS   GUINEA PIGS

IMMUNE SERUM   ANTI VIRION SURFACE   ANTI VIRION CORE
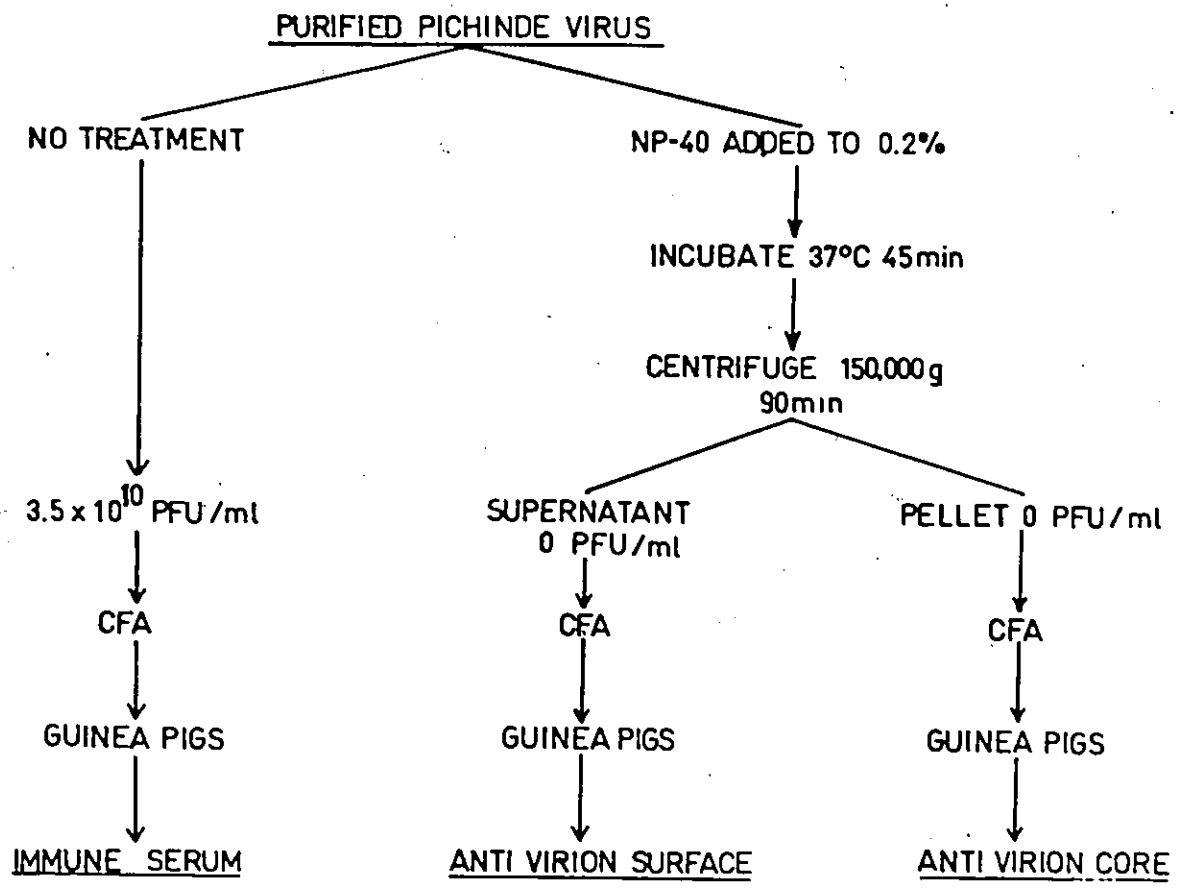
Figure 26.

        Immunodiffusion test showing identity
between antigens detected in lysates of infected
cells (CELL LYS) by immune serum (IMM S), and anti-
sera against NP-40 obtained virus "cores" (ANTI
CORE) and partially purified CF antigen (ANTI AG).
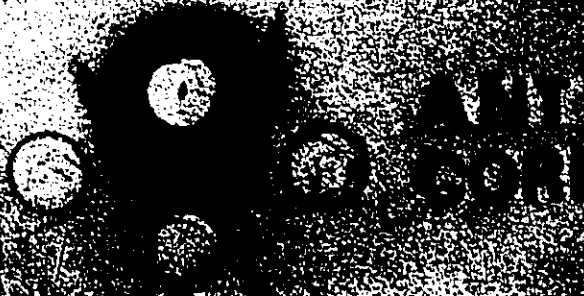
staining of the cytoplasm (Figure 27, 28). Antiserum directed against detergent solubilized material gave a different pattern of staining. With this antiserum vacuolar inclusions of antigen were observed in the cytoplasm of infected cells (Figure 29).

Table XII summarizes the reactivity of the antisera against virion components as assessed by CF, immunodiffusion, and indirect immunofluorescence.

E. Association of Antigens with the Virion as Detected by Immunodiffusion

Figure 30 shows the immunodiffusion reaction obtained when purified Pichinde virus was disrupted with NP-40, ether, and ribonuclease then reacted against immune serum and against antiserum specific for the major CF antigen. Note that 2 precipitin bands formed between the wells containing immune serum and infected cell lysates. These bands are continuous with those detected in the disrupted virus preparation. Antiserum against the major CF antigen also produce a band of identity between the disrupted virus and infected cell lysate wells. Ribonuclease digestion of the NP-40 disrupted virus was necessary for the development of sharp precipitin bands in this reaction.

Figure 27

     Indirect immunofluorescent stain of
Pichinde virus-infected Vero cells by antiserum
against intact virus. Antiserum was obtained as
described in Figure 25. Top, infected cells;
bottom, control cells.
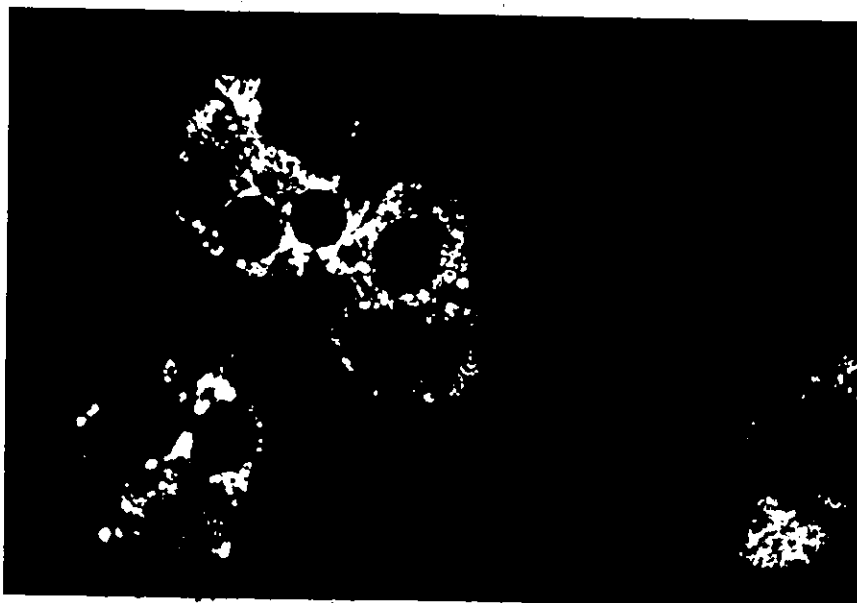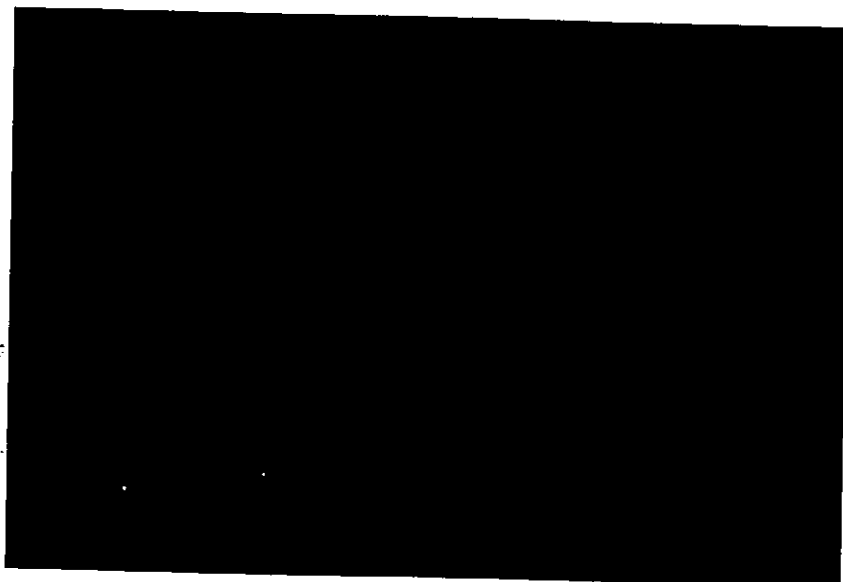
Figure 28

Indirect immunofluorescent stain of Pichinde
virus-infected Vero cells by antiserum against the
NP-40 insoluble virion "core" fraction. Top,
infected cells; bottom, control cells.
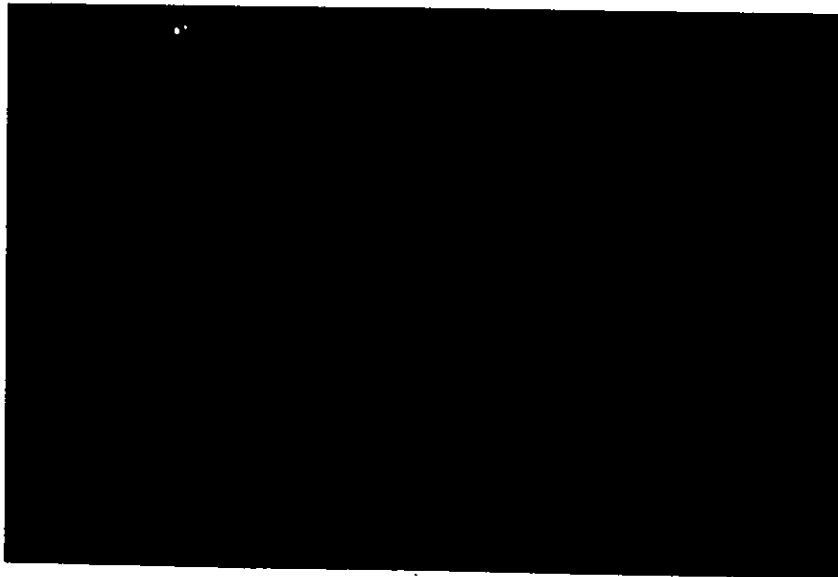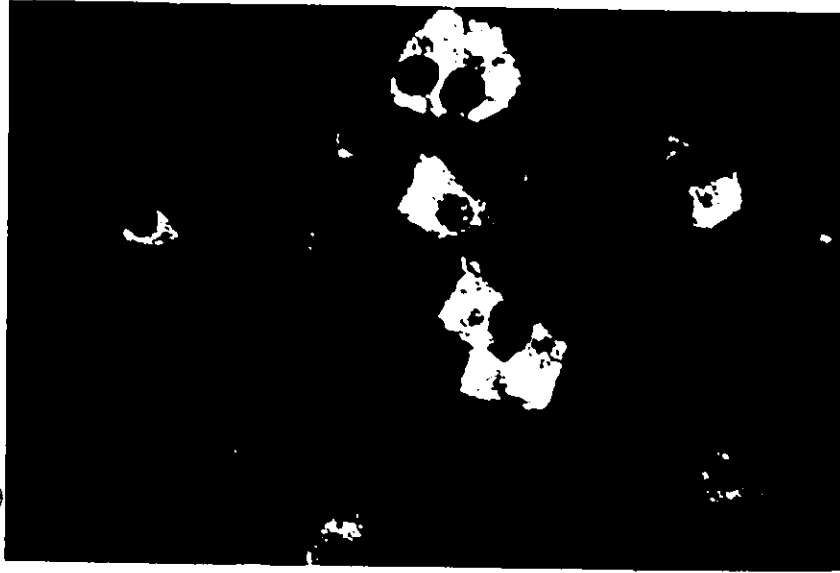
Figure 29

Indirect immunofluorescent stain of Pichinde
virus-infected Vero cells by antiserum against
the NP-40 soluble virion component.

TABLE XII

IMMUNOLOGIC REACTIVITY OF GUINEA PIG ANTISERA RAISED AGAINST INTACT PICHINDE VIRUS AND
NP-40 DISSOCIATED VIRAL COMPONENTS

| | | | Antibody Reaction | |
Immunogen[1]	Animal No.	CF Antibody Titer[2]	Immunodiffusion[3]	Immunofluorescence[4]
Intact virus	1	1:64	2 Bands	Positive granular cytoplasmic staining and reticular surface reaction.
	2	1:128	2 Bands	
NP-40 disrupted virus – Insoluble core fraction	1	1:32	1 Band (major)	Positive granular cytoplasmic staining
	2	1:64	1 Band (major)	Negative surface.
NP-40 disrupted virus solubilized fraction	1	<1:2	Negative	Positive vacuolar cytoplasmic staining
	2	1:8	Negative	

[1] All immunogens were emulsified in complete Freund's adjuvant.

[2] Complement-fixing antibody titers 22 days after inoculation.

[3] Immunodiffusion reaction of 22 and 28 day sera against lysates of BHK_{21} cells infected with Pichinde virus.

[4] Indirect immunofluorescent staining of Vero cells infected with Pichinde virus.

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Figure 30

Immunodiffusion reaction showing identity
of antigens detected in lysates of infected BHK_{21}
cells (CELL LYS) with antigens detectable in
disrupted Pichinde virus (DIS V). Sera used were
Pichinde-immune guinea pig serum (IMM S) and mono-
specific antiserum against the heat stable CF
antigen (ANTI AG).
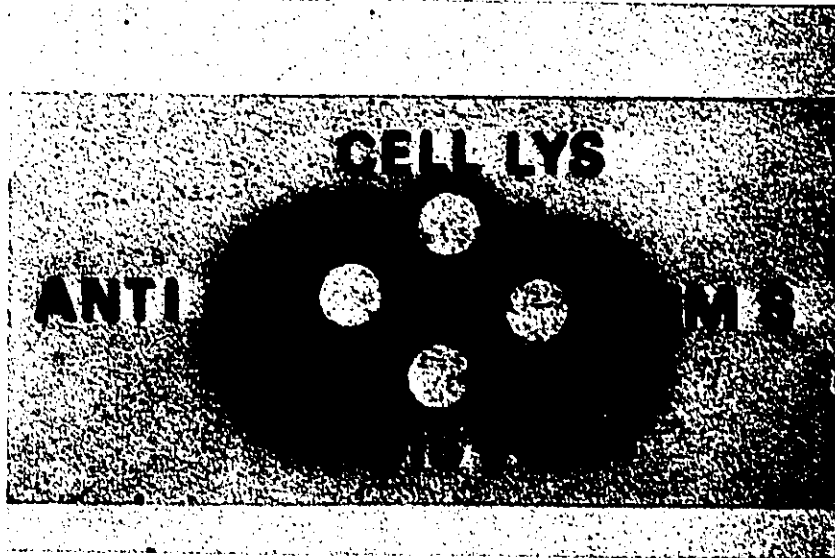
Antigenicity detectable by CF was also liberated when purified virus was disrupted with NP-40. In one experiment, a purified virus preparation containing approximately 200 ug/ml of protein was tested before and after NP-40 disruption. Prior to disruption the CF antigen titer was 1:4, while after detergent treatment the titer was 1/16. From these experiments it appeared that the major and minor CF antigens were virion components.

F. Relationship of Antigenic Activity with Sub-
structures of Pichinde Virus

The previous experiments demonstrated that the CF antigens were associated with NP-40 liberated components of the Pichinde virion. To better characterize this association, a series of fractionations of purified virus were carried out. Purified virus was disrupted with NP-40 as described previously, then layered onto a 20-50% w/w sucrose gradient containing 0.1% NP-40 and centrifuged for 2 hr at 35,000 RPM. Table XIII shows the distribution of radiolabelled protein and RNA obtained in the material which pelleted through the gradient and the soluble material which remained at the top of the gradient. No radiolabel was found in the middle fractions of the gradient. The insoluble material pelleted through sucrose with a density of 1.24 g/ml and

TABLE XIII

DISTRIBUTION OF ANTIGENICITY AND RADIOLABELLED VIRAL PROTEIN AND RNA OBTAINED WHEN

PURIFIED PICHINDE VIRUS WAS DISRUPTED WITH NP-40 AND CENTRIFUGED ON A 20-50% SUCROSE

GRADIENT CONTAINING 0.1% NP-40

Gradient Fraction	Antigenicity[1]		Protein		RNA	
	Major	Minor	CPM[2]	% of Total	CPM[3]	% of Total
Soluble material P≤ 1.05	+[4]	+	1780	72%	360	46%
Middle fractions P= 1.16 - 1.18	-	-	nil[5]	nil	nil	nil
Pelleted fraction P= >1.24	+	+/-	690	28%	400	53%

1 Antigen bands detected by immunodiffusion.

2 CPM ^{3}H amino acid label/50 µl aliquot.

3 CPM ^{14}C uridine label/50 µl aliquot.

4 + Indicates presence of immunodiffusion band.

5 Nil indicates counts not significantly above background.

contained 53% of the labelled RNA and approximately 28% of the protein. The soluble material at the top of the gradient contained 46% of the labelled RNA in addition to 72% of the protein. Both fractions contained antigencity detectable by CF and immunodiffusion.

Figure 31 shows the results obtained when the two virion fractions were analyzed for their polypeptide content by SDS-PAGE. The NP-40 solubilized fraction was found to contain all 4 of the virion polypeptides in approximately the same proportions as intact virus. The NP-40 insoluble fraction contained enriched amounts of the V_{II} and V_{IV} polypeptides, decreased proportion of the V_I polypeptide, and no detectable V_{III}.

The requirement for intact RNA for integrity of the insoluble particle was demonstrated by digestion of the particle with RNase. Figure 32 shows the results of a typical experiment in which the pellet fraction from a 20-50% sucrose -0.1% NP-40 gradient was digested with 100 ug/ml RNase then re-centrifuged on a 5-20% sucrose gradient containing 0.1% NP-40. Treatment with RNase rendered a portion of the radiolabelled protein soluble. This material, sedimenting at approximately 4S, contained all of the CF antigen activity previously associated with the NP-40 insoluble particle. Analysis by immuno-

Figure 31

        Polypeptide composition of soluble and

insoluble components obtained from Pichinde virus

after NP-40 disruption.   Purified Pichinde virus,

radiolabelled with ^{3}H-amino acids, was disrupted

with 1% NP-40 (15', 37°C) then layered onto a

20-50% w/w sucrose gradient containing 0.1% NP-40

and centrifuged for 90 min at 30,000 RPM. The

soluble material, remaining on top of the gradient,

and the insoluble material, pelleting through the

gradient were harvested, adjusted to 1% SDS, 0.1%

2-mercaptoethanol and 0.5 M urea and prepared for SDS-

PAGE. ^{14}C-amino acid labelled Pichinde virus

(1500 CPM) was mixed with approximately 3000 CPM

of the sample, and the mixture was co-electrophoresed

on a 15% polyacrylamide gel containing 0.2% SDS and

0.5 M urea.   (●—●) ^{3}H CPM in sample (- - - - -)

^{14}C CPM in virus marker.
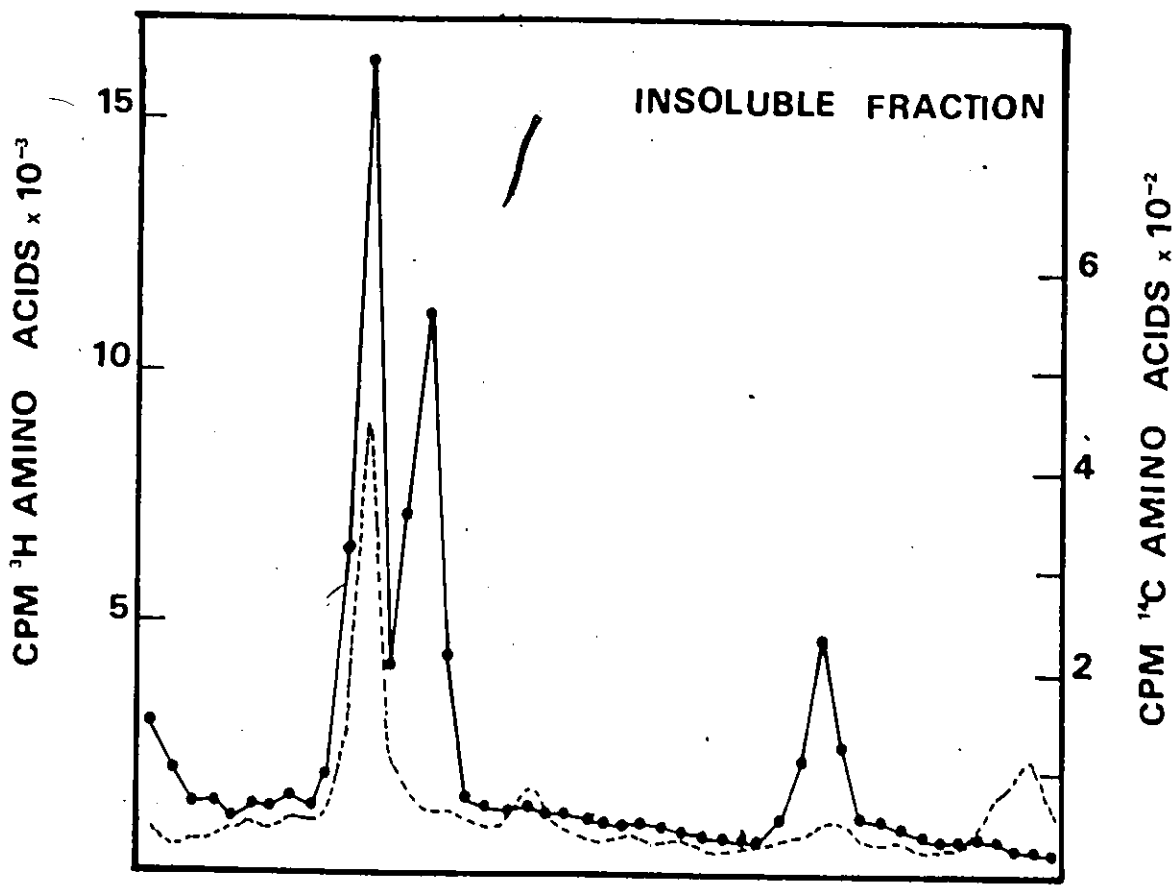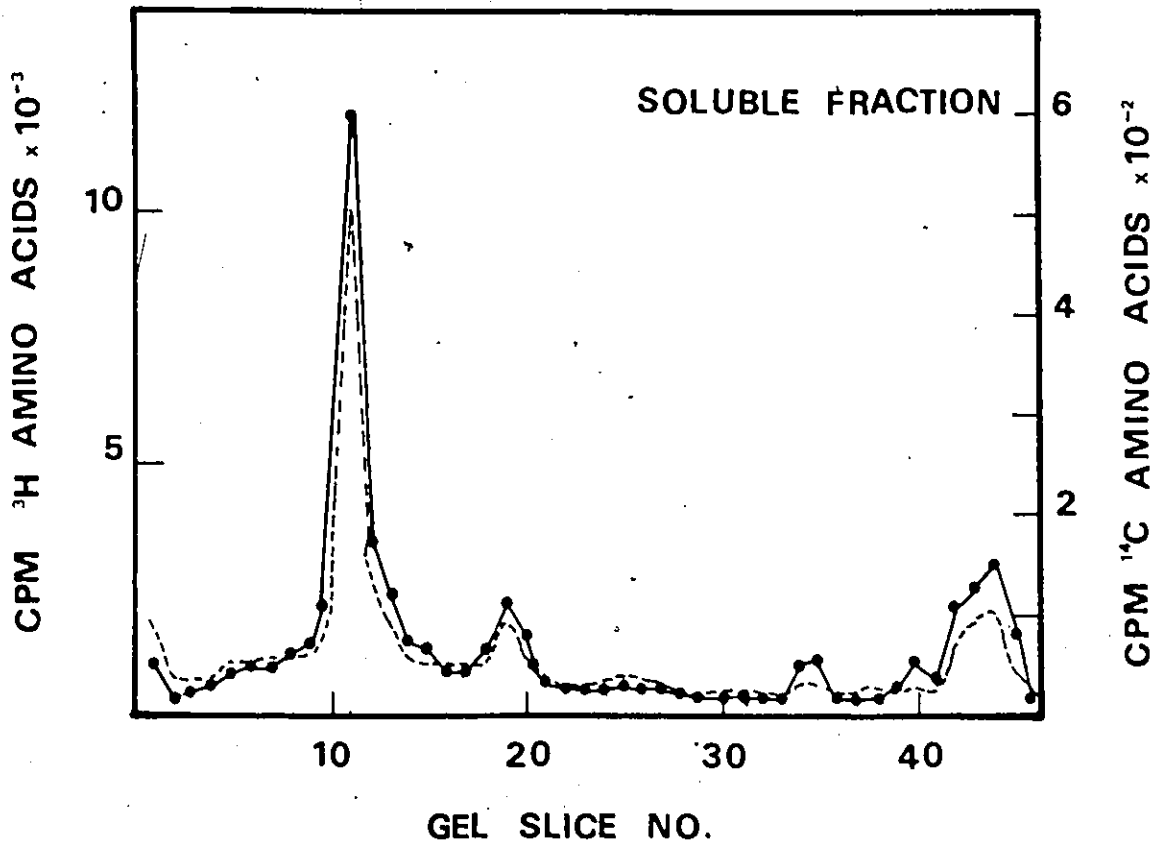
Figure 32

Soluble antigen liberated by treatment of

virion core component with RNase. The core component

was obtained by NP-40 treatment of ^{3}H-amino acid

labelled virus (see legend Fig. 31) then incubated

with 100 ug/ml RNase for 30 min at 37°C. After

incubation, the material was layered onto a 5-20%

sucrose gradient containing 0.1% NP-40 and centrifuged

for 22 hr at 35,000 RPM. Fractions were collected and

assayed for CF antigen (●--●) and ^{3}H CPM (O—O).
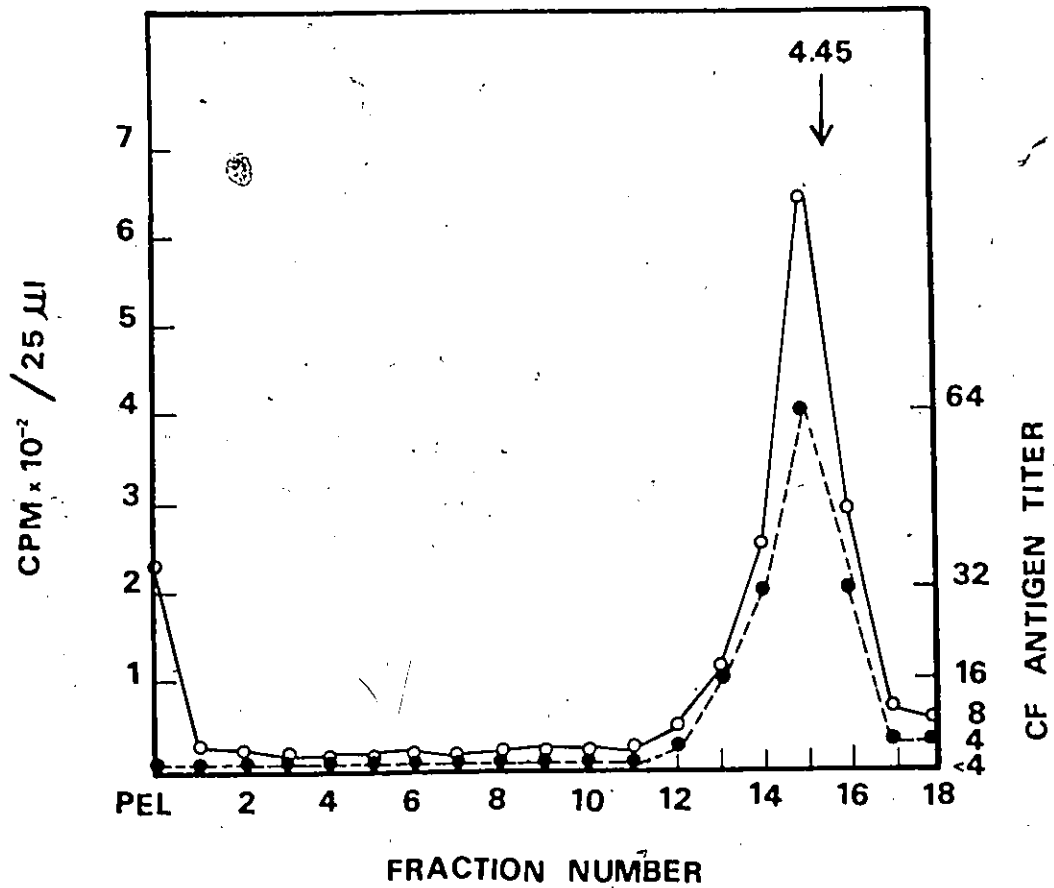
diffusion of the 4S fraction revealed the presence of
two precipitin bands which identified with the major
and minor bands found in lysates of infected cells
(Figure 33). The 4S peak was shown by SDS-PAGE to contain
polypeptides migrating with the V_{II} glycoprotein and
close to the V_{IV} polypeptide as well as a reduced amount
of the V_I polypeptide (Figure 34). The material which
sedimented through the 5-20% sucrose gradient contained
no detectable antigenicity and contained only the V_I
polypeptide. Table XIV summarizes the distribution of
radiolabelled protein in each polypeptide through the
fractionation series. Note that the loss of the V_{III}
glycopeptide and the V_I polypeptide did not result in
loss of antigenic activity detectable by CF or immuno-
diffusion.

G. Comparison of the Polypeptide Composition of
   Purified Major Antigen Derived from Infected Cells
   with the Virion Polypeptides

   Major antigen, labelled with ^3H amino acids, was
purified as described above (Table XI) then concentrated
by 15% TCA precipitation and compared with viral poly-
peptides by SDS-polyacrylamide gel electrophoresis.
Comparisons were made by coelectrophoresis of ^3H-amino
acid labelled antigen with ^{14}C amino acid labelled poly-
peptides obtained from purified virus, or with ^{14}C amino

Figure 33

    Immunodiffusion test of virion components
obtained by NP-40 solubilization and RNase digestion.
The soluble (1) and insoluble (2) fractions obtained
from purified Pichinde virus by treatment with
NP-40, and the soluble antigen-containing (3) and
insoluble (4) fractions obtained from the virus
"cores" by RNase digestion were tested against immune
guinea pig serum (IS) by immunodiffusion. Soluble
antigen (AG) derived from infected cells, and purified
virus, disrupted with NP-40 and RNase (V), were
included for reference.
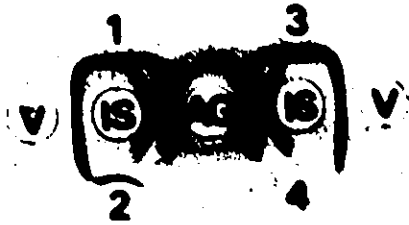
Figure 34

        Polypeptides present in the soluble antigen

liberated from virion "cores" by RNase treatment.

The soluble, antigen-containing fraction from

virion "cores" was prepared as described in figure 32.

The gradient fraction containing the peak CPM and

antigen was analyzed by SDS-PAGE as described in

figure 31. 2,000 CPM of sample radiolabelled with

^{3}H-amino acids (●——●) was coelectrophoresed with

1,500 CPM of ^{14}C amino acid labelled Pichinde virus

marker (--ᵣ--).
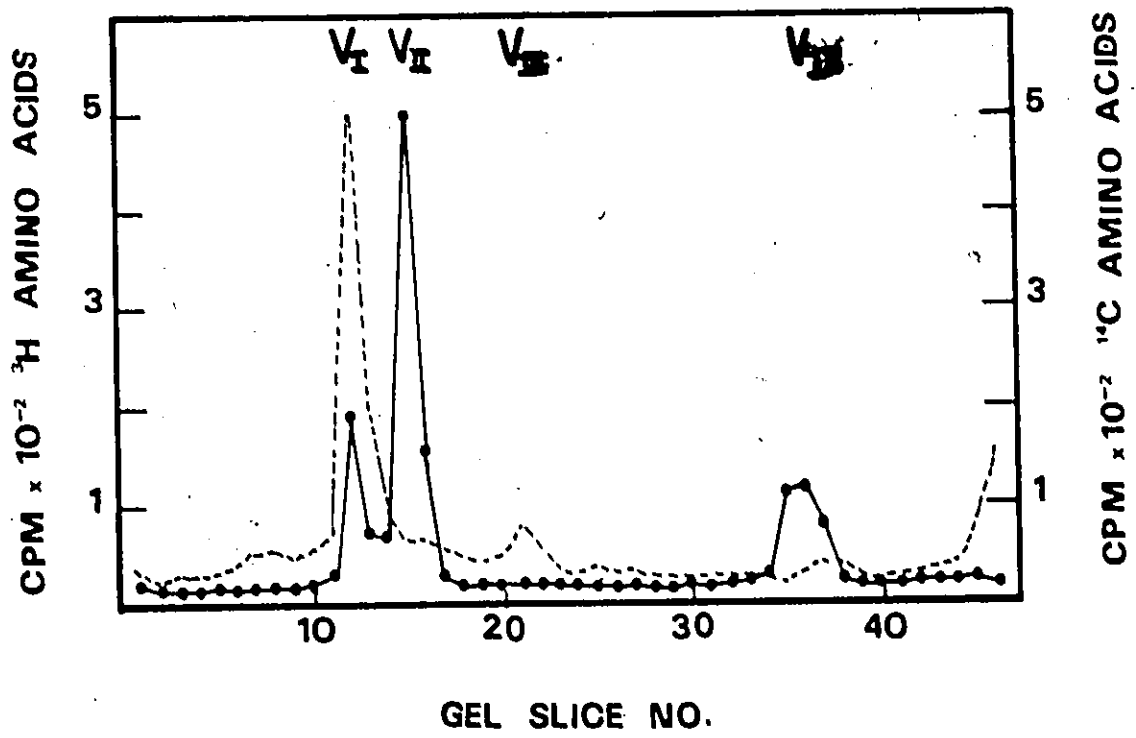
TABLE XIV

RELATIVE DISTRIBUTION OF THE FOUR MAJOR VIRION STRUCTURAL POLYPEPTIDES AFTER

NP-40 SOLUBILIZATION AND RNase DIGESTION

Treatment of purified virus	Ratio of Amino Acid Labelled Viral Polypeptides[1]			
	V_I	V_{II}	V_{III}	V_{IV}
None	71%	10.4%	13.8%	4.8%
NP-40 Solubilization				
Soluble (72%)[2]	70%	8.4%	16.7%	5.3%
Insoluble "Core" (28%)	46%	39.5%	0	14.4%
RNase treated core run on 5-20% sucrose gradient				
4S fract. (85%)	16%	60%	0	24%
Pellet (15%)	92%	8%	0	0
Immune precipitin of 4S fract. (96%)[3]	18%	62%	0	19.6%

[1] The percentage of CPM residing in each individual polypeptide was determined relative to the total CPM in V_I, V_{II}, V_{III} and V_{IV}.

[2] The number in parentheses represents the relative distribution of the CPM in each of the fractions obtained.

Continued

3. Amino acid labelled 4S fraction was reacted with 1:32 diluted immune hamster serum for 18 hr at 4°C then 1:8 diluted antibody to hamster IgG was added. After 2 hr at 37°C the precipitate was collected, washed three times, then solubilized in 1% SDS and prepared for polyacrylamide gel electrophoresis. Normal hamster serum (1:32) precipitated 4% of the counts.

acid labelled, parallel purified control cell material.
Figure 35 shows the pattern of radiolabel obtained.
Two low molecular weight polypeptides unique to the
preparation of purified antigen were observed; control
material contained no appreciable radiolabel in the
corresponding fractions. Coelectrophoresis with purified
virus revealed that neither of the two polypeptides
present in the antigen preparation co-migrates exactly
with any of the virion structural polypeptides (Figure 36).

A potential explanation for this observation is
that either one or both of the low molecular weight
polypeptides arises through proteolytic cleavage of the
V_{II} glycopeptide. To test this hypothesis, antigen was
purified which was radiolabelled in the glycoprotein
with ^{3}H-glucosamine. Although glucosamine label was
present in the first two peaks eluting from the Biogel
P-200 column (cf. Fig. 22), significant amounts of
glucosamine label were not found in the Biogel P-200
column fractions which contained CF antigen activity.

H. Purification of Viral Polypeptide by Isoelectric
   Focusing

Isoelectric focusing was explored as a means of
isolating viral proteins in order to determine their
relationship to the cell associated antigens. Approximately
3 milligrams of viral protein was disrupted with NP-40,

Figure 35

Polypeptide composition of purified cell-
derived major CF antigen. Major antigen, labelled
with ^{3}H-amino acids was purified by rate zonal
centrifugation, isoelectric focusing and Biogel
P-200 gel filtration. The purified antigen was pre-
cipitated with 15% TCA, then washed with 5% TCA and
acetone and dried. The dried protein was solubilized
and electrophoresed as described in figure 31.
(●—●) antigen purified from infected BHK_{21} cells.
(○—○) parallel purified ^{14}C amino acid labelled
control material.
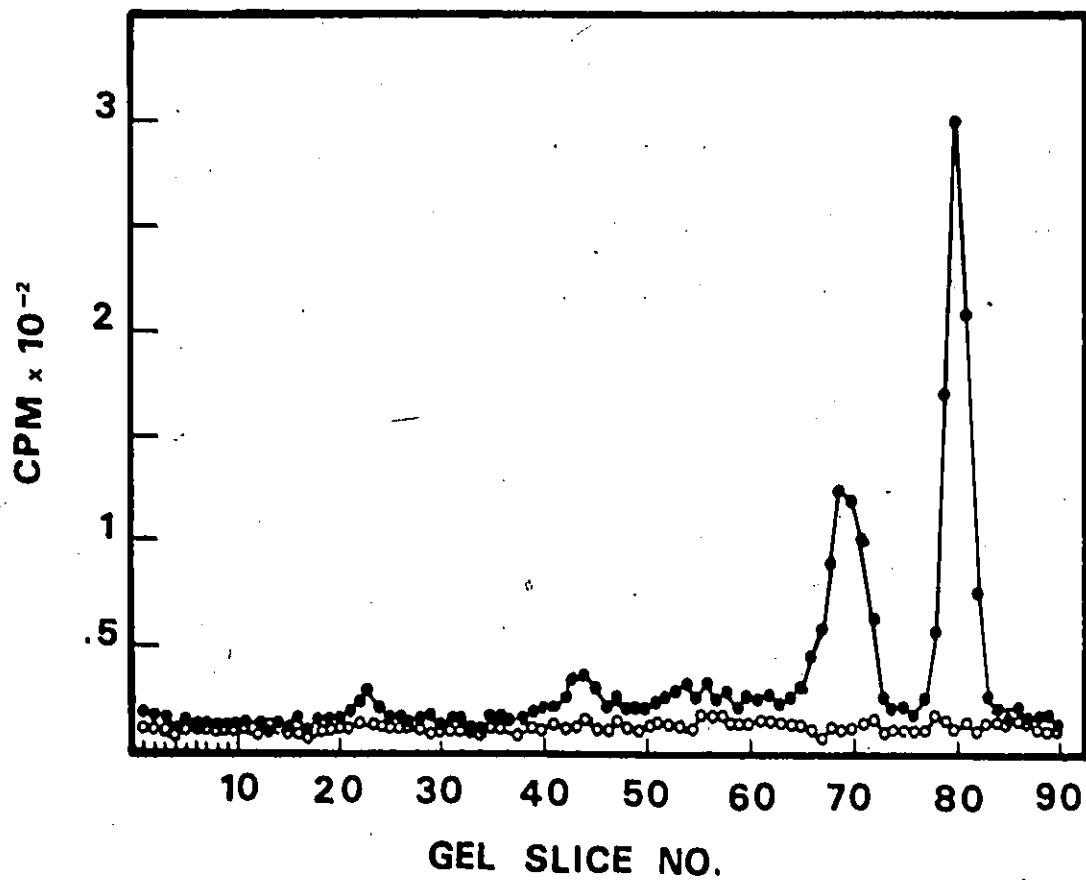
Figure 36

 Comparison of the polypeptides in purified
antigen with the virion structural polypeptides.
Antigen radiolabelled with ^{3}H-amino acids was
purified as described in Fig. 35, then mixed with
1,500 CPM of ^{14}C amino acid labeled Pichinde virus
marker and electrophoresed as described in figure 31.
(●—●) purified antigen; (O—O) ^{14}C amino
acid labelled virus marker.
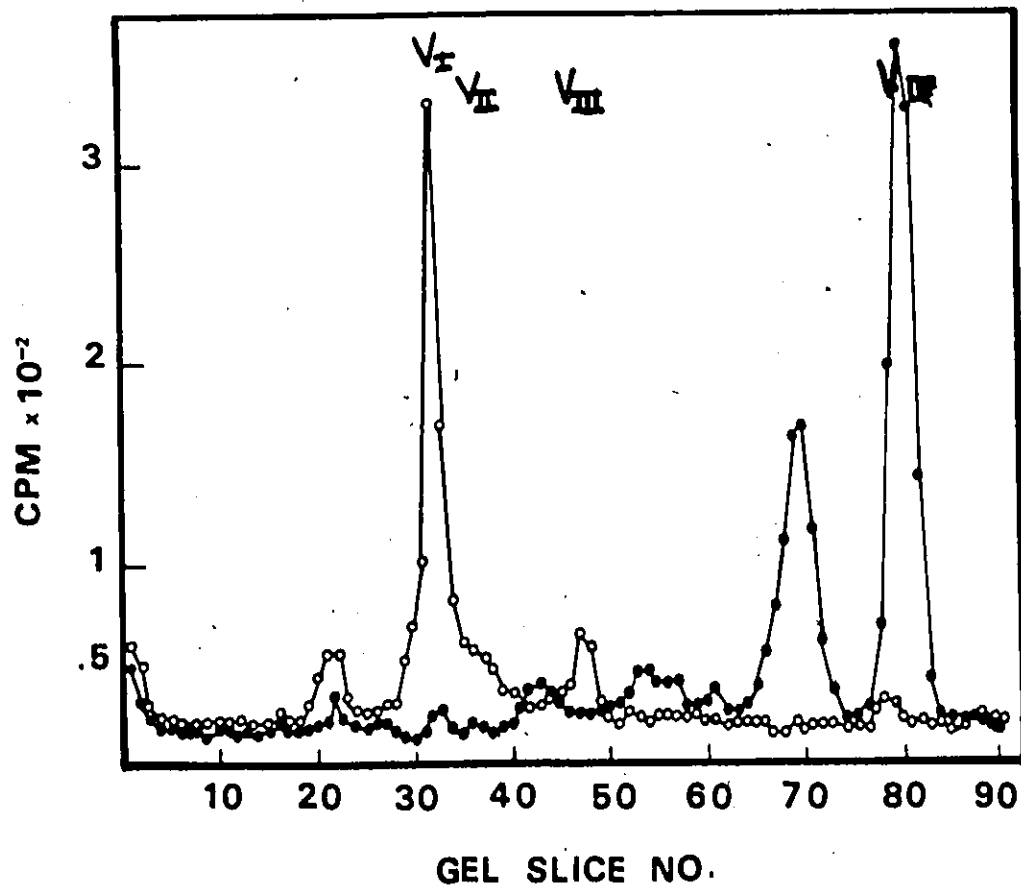
ether and RNase and loaded onto a pH 3.5-10 iso-
electric focusing gradient as described in Materials
and Methods. After focusing for 96 hr at 300 volts,
fractions were collected and assayed by CF, and immuno-
diffusion for antigen and for the radioactivity
present in each fraction. Figure 37 shows the pattern
of radiolabelled viral protein and CF antigenicity
obtained in this experiment. Three peaks of protein
were obtained at pH values of 4.3, 6.4, and 8.9. Of
these only the pH 6.4 and 8.9 peaks were associated
with CF antigenicity. The pH 4.3 peak was composed
of protein that was visably precipitated, and may have
been denatured. Analysis by SDS-PAGE of three protein
peaks yielded the following information: ·The pH 4.3
peak contained all 4 of the virion polypeptides in
proportions similar to those found in the virion
(Figure 38). The pH 6.4 peak contained the V_I poly-
peptide and the V_{II} polypeptide (Figure 39). The peak
of protein focusing at pH 8.9 did not contain sufficient
counts of radioactivity for definitive analysis by PAGE.
By immunodiffusion the pH 6.4 peak was shown to contain
both the major antigen and the minor antigen.

Figure 37

     Isoelectric focusing of components of purified
Pichinde virus. 3 mg of purified virus radiolabelled
with ^{3}H-amino acids was disrupted with 1% NP-40,
ethyl ether, and 100 ug/ml RNase, then loaded into
an isoelectric focusing gradient containing 0.1%
NP-40. After 96 hr of focusing at 300 v, fractions
were collected and assayed for CF antigen (●—●)
and ^{3}H-CPM (·——·). The pH of the fractions at 4°C
was also determined (− − − −).
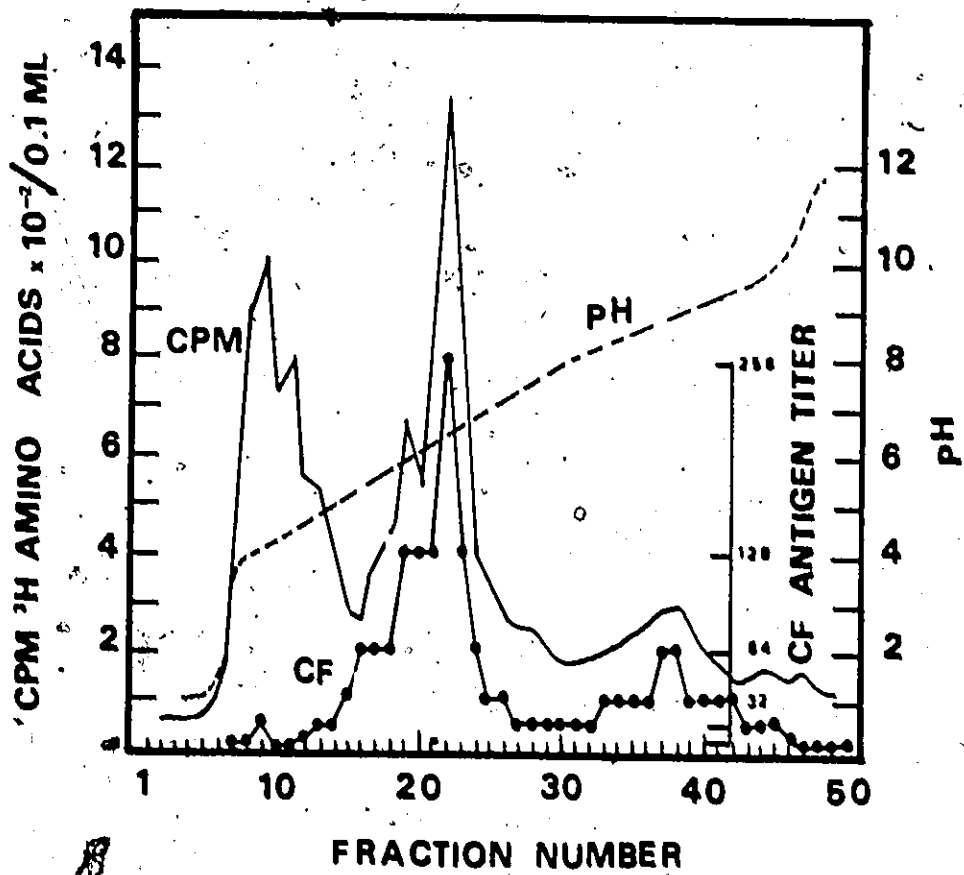
Figure 38

        Polypeptide composition of the pH 4.3 protein

peak. Fractions containing the peak of amino acid

label focusing at pH 4.3 (see Fig. 37) were pooled,

and an aliquot was precipitated with 15% TCA, then

washed with 5% TCA and cold acetone. The precipitated

material was solubilized for SDS-PAGE and co-

electrophoresed on a 15% acrylamide gel with ^{14}C

amino acid labelled virus marker. (●—●) sample,

(—·——) virus marker.
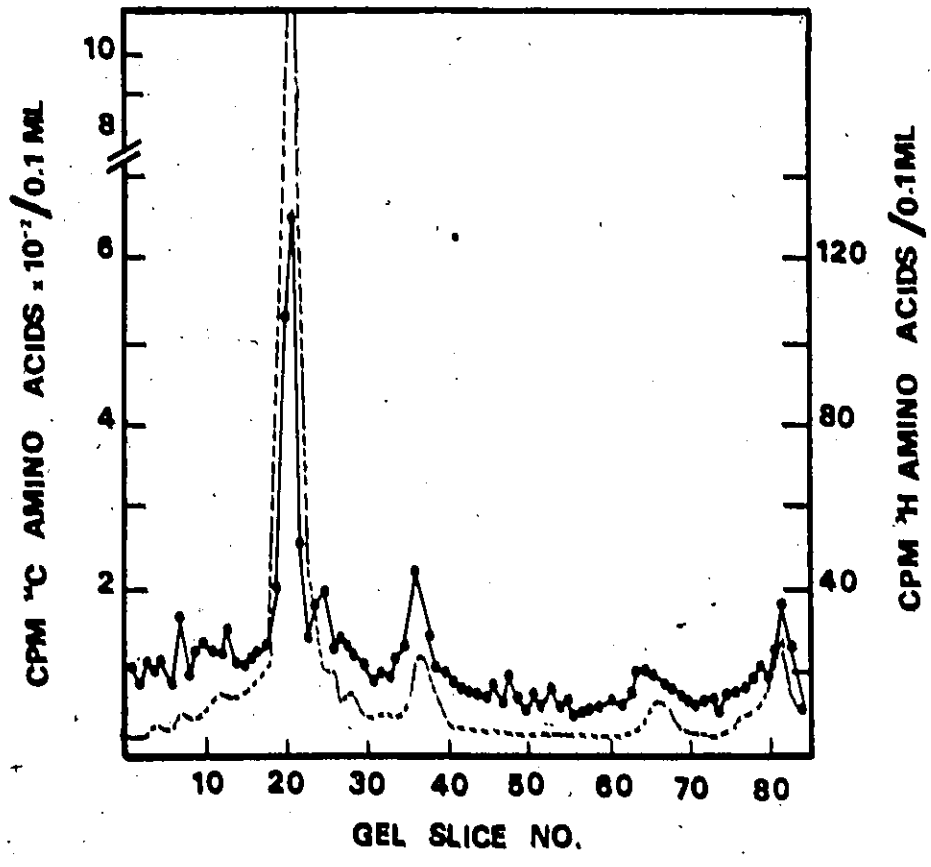
Figure 39

Polypeptides present in the antigen-
containing protein peak obtained at pH 6.4 after
isoelectric focusing of purified Pichinde virus.
The peak of amino acid label focusing at pH 6.4
was electrophoresed on a 15% acrylamide gel as
described in Fig. 38 (●—●) sample, (- - - - -)
virus marker.
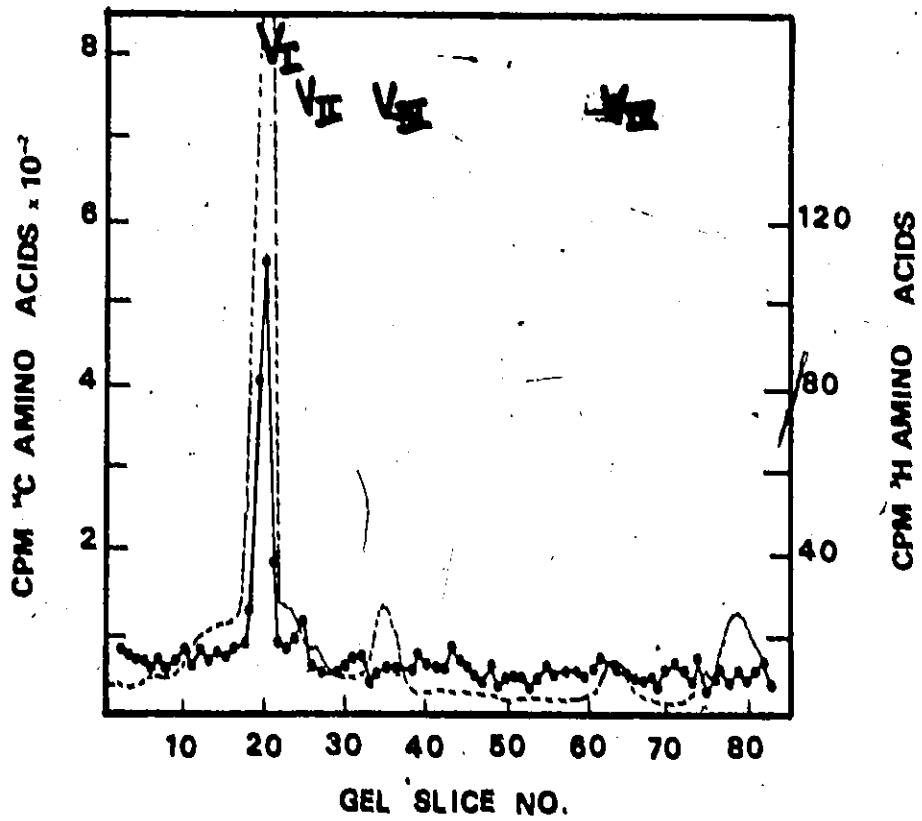
DISCUSSION

The arenaviruses provide an excellent model
for examining persistent virus infections. In nature,
these viruses produce persistent infections in a
limited number of rodent species; each virus being
associated with a single rodent species. Persistent
infections with Pichinde virus have been demonstrated
only in Oryzomys albigularis (Trapido and Sanmartin,
1971). However, it is apparent that Pichinde virus can
infect a broad range of host species. During the
course of this study and a previous study (Trapido and
Sanmartin, 1971), the virus was shown to infect hamsters,
mice, guinea pigs and rabbits. Virus persistence did
not appear to have occurred in any of these animals.
Thus, persistence of Pichinde virus appears to be species
specific.

Whether or not Pichinde virus infection is
limited to mammals is not known. The only species
in which I was unable to show evidence of infection was
the chicken (Data not shown). In their original report,
Trapido and Sanmartin described several virus isolations
from mites recovered from viremic O. albigularis rodents.
These mites did not survive well in the laboratory, so
that long term observation was impossible. These workers

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could not eliminate the possibility that the virus
isolated reflected surviving virus in engorged viremic
blood.

Humans are susceptible to a number of arena-
viruses and infections have occurred following exposure
to these viruses in the laboratory. The hazardous
nature of some arenaviruses including Lassa virus
(Leifer et al, 1970), LCM virus, (Smadel et al, 1942;
Scheid et al, 1956; Baum et al, 1966; Parker et al, 1976),
and Machupo virus (Peters et al, 1973) to laboratory
workers has been well documented. Pichinde virus has not
been shown to cause naturally occurring disease in
humans (Trapido and Sanmartin, 1971; Johnson et al, 1973).
The potential of Pichinde virus to infect humans in a
laboratory setting was unknown at the outset of this
study. For this reason, the seroepidemiological survey
of Pichinde virus infection among laboratory workers was
done (Buchmeier et al, 1974). The results of this study
indicate that in the laboratory, exposure to highly con-
centrated Pichinde virus can result in infection in humans.
Apparently this capability is dose related, as those workers
exposed to known high concentrations (solutions of
$>10^{10}$ PFU/ml) of virus showed the highest percentage of
seroconversion. The infections did not appear to be

contageous among humans since there was a lack of
evidence of infection among those in the moderate
risk group who had close contact with the infected
individuals.

No definite illness could be associated with
the Pichinde virus infection. In a single worker where
the infection could be traced to a 1 month period,
mild dysuria, myalgia, and a localized vesicular
eruption of one hand were the only symptoms noticed.
Although no illness was attributed to the infection in
healthy laboratory workers, the potential to cause
illness under mitigating circumstances such as immuno-
suppression, pre-existing illness or pregnancy is not
known. However, the data suggest that Pichinde virus
can infect humans and that these infections are generally
asymptomatic. This is in contrast to infections with
certain other arenaviruses which may produce febrile
illness associated with meningitis (LCM virus), hemorrhagic
fever (Junin and Machupo viruses) or edema and liver
lesions (Lassa virus). Thus, among the arenaviruses
there is considerable variability in the pathogenesis of
diseases which may develop after infection of an incidental
host such as the human.

The influence of host factors on the outcome of infection by the arenaviruses can be further illustrated by the data derived from infecting hamsters with Pichinde virus. The lethal effects of infection of suckling hamsters with the viruses of the Tacaribe complex have long been known. Initial isolations of many of these viruses were accomplished by intracerebral inoculation of suckling hamsters with materials obtained in the field (Webb et al, 1970; Trapido and Sanmartin, 1971), and all of the agents of the group are pathogenic for these animals. In contrast to the demonstrated pathogenicity for newborn hamsters is the reported lack of pathogenic effect of Tacaribe complex viruses on adult hamsters (Johnson et al, 1973).

The resistence to the lethal effect of Pichinde virus in LVG (outbred golden Syrian) hamsters was found to develop between 6 and 8 days of age. Earlier the observation was made that cyclophosphamide immuno-suppresion of hamsters infected at 7 days of age rendered them as susceptible as newborns to Pichinde virus infection (G.H. Houlditch, personal communication). This suggested that the disease was basically a virus-induced pathology and not an immune pathology as previously described in LCM virus infection of the mouse (Oldstone and Dixon, 1969, 1970; Gilden et al, 1972a, b).

Virus replication _in vivo_ was studied relative to the age at the time of inoculation of hamsters, and a marked age-dependence of virus titers was observed. When 3 to 12 day old hamsters were examined, the highest titers were always observed in the blood and organs of the youngest animals tested. Histopathological examination of organs from newborn and adult LVG hamsters infected with Pichinde virus revealed renal necrosis with massive tubular destruction and hemorrhage in the newborn. Minimal pathologic changes were seen in the kidneys of the adult LVG hamsters. Superimposed upon the renal destruction in the newborns were hemorrhages into the brain parenchyma and focal hepatic lesions. Taken individually, any of these conditions could account for the fatal outcome of infections in the newborn, however, death was probably due directly to the kidney lesions (Dr. F.A. Murphy, personal communication). Some evidence of an infiltrative host response was evident in the infected kidneys and brains, but the contribution of this inflammation to the overall pathology was probably minimal. These observations suggest that the fatal outcome of Pichinde virus infection in the newborns was related to a virus-induced pathology and that one or more factors affecting virus replication

and leading to resistence develop with age.

I found differences in susceptibility to Pichinde virus infection among adult hamsters of different strains. Adult hamsters of the MHA inbred strain were found to be susceptible to lethal Pichinde virus infection. The susceptibility was observed over a wide range virus doses from 35 to 3.5 X 10^6 PFU, and the animals usually died between 12 and 21 days after infection, somewhat later than the infected newborns. The MHA hamsters were found to have significantly higher levels of virus in the blood at 8-11 days after infection than LVG hamsters which rarely showed ill effects of the virus. Further, the virus titers appeared to be similar during the first 4 days of the infection, but differed after 4 days. This suggested that some factor or factors operative in limiting the infection in the LVG animal was deficient in MHA adults. Confirmatory results were obtained by examination of kidneys, spleen and brain of each strain between 1 and 15 days after infection. In each case the highest titers were found in the MHA hamsters.

Early peaks of virus titer were observed in the spleens of MHA and LVG hamsters at 8 days after infection. It is significant that the virus titer

in the MHA spleens remained at a high level ($>10^8$ PFU/ gm) throughout the duration of the 15 day study period, whereas the titers in the LVG spleens dropped during this period. This may reflect a lymphoreticular tropism similar to that reported by Murphy et al (1976) in Tamiami virus infection.

Virus titers in the brains of infected animals offered another interesting comparison. While virus was not detectable in the brains of infected LVG hamsters, MHA hamsters brains titered $10^{5.6}$ and $10^{6.6}$ PFU/gm at 8 and 11 days respectively. This observation was supported by the histologic observation of necrotic lesions in MHA brains at these times, but complete absence of pathology in the LVG brains examined. Apparently the virus was able to penetrate the blood-brain barrier in the MHA but not the LVG hamster.

Adult MHA hamsters displayed a massive focal destruction of the liver parenchyma. Additional, less severly affected target organs included the kidneys and brain. The necrotic lesions observed were interesting in that there was a lack of significant clearance of necrotic debris; this debris appeared to accumulate in and around foci.

The disease in the LVG hamsters was similar
in character but quantitatively less severe. In these
animals the necrotic lesions were limited to the
kidney and liver, and were fewer in number. The
necrosis again appeared to lack significant inflammation.

.The differences in the susceptibility to
Pichinde virus infection in MHA and LVG hamsters did not
appear to reside at the cellular level. Primary kidney
cell and macrophages from either strain supported virus
replication equally well in vitro. Thus the differences
observed in vivo appeared to result from differences
in control or clearance of the infection and not simply
from a lack of replicative potential in the LVG
strain.

The results of experiments using the immuno-
suppressant cyclophosphamide suggest that the control
of replication is related to immunological factors.
Cyclophosphamide (100-150 mg/kg) given 3 days after infection
was not found to be protective to MHA hamsters infected
with Pichinde virus. On the contrary, the susceptibility
of both MHA and the normally resistant LVG animals was
increased by immunosuppression. This was taken as
evidence that the disease was limited by immunologic
factors which were blocked by cyclophosphamide. The

finding that CF antibody levels measured 30 days after infection were not greatly depressed in drug treated hamsters suggests that humoral immunity was not responsible for virus elimination, however, the CF antibody may have little or no capacity to neutralize the virus.

The susceptibility of several other inbred hamster strains to Pichinde virus infection was tested. Although the results with respect to one strain, CB, were not consistent, it appears that most of the strains tested were resistent while the MHA strain was clearly susceptible. Within the MHA strain mortality typically ran from 60-90% within 25 days after infection. Essentially all of the MHA animals infected became noticably ill with symptoms of dehydration, emaciation, irritability, diarrhea and facial edema appearing around 6-8 days after infection followed terminally by loss of responsiveness to stimuli and difficulty in breathing.
In those animals where the disease did not result in death, the animals exhibited the early symptoms for a variable period and then usually improved. Such hamsters were noticably smaller than their age matched controls, and were usually somewhat more irritable for a month or more following recovery.

Similarity exists between Pichinde virus infection in the adult MHA hamster and Lassa fever in humans. In Lassa fever, liver necrosis has been reported which may bear some similarity to that observed in Pichinde infected hamsters (Winn et al, 1975). These comparisons await further examination of both diseases in order to be validated.

There is little similarity between Pichinde virus disease in adult hamsters and LCM disease in the adult mouse. Whereas LCM disease is marked by massive infiltration of the meninges and in some cases, the infected viscera, Pichinde virus disease shows little infiltration of infected tissues. In addition, cyclophosphamide treatment, known to induce chronic LCMV carrier mice (Gilden et al, 1972a), increased the severity of Pichinde virus disease.

The observations of age dependence of pathogenesis of Pichinde virus infection are in sharp contrast to the reported studies of LCM virus. In LVG hamsters, death is observed in the newborn but not in the adult after Pichinde virus infection, whereas in LCM virus infection of mice, the opposite is observed. The degree of pathology after Pichinde virus infection appears to be related to virus replication in vivo. Comparing adult MHA and LVG hamsters revealed an association between

fatal infection in MHA hamsters and high titers of virus in the tissues. Immunosuppression with cyclophosphamide, a procedure which has been demonstrated to save LCMV infected adult mice from death (Gilden et al, 1972a), renders normally resistant LVG hamsters susceptible to lethal infection by Pichinde virus.

Nathanson et al (1975) have reported necrotic disease following LCM and Tacaribe virus infections in the rat during the first 4 days of life. These lesions are confined to the brain and retina in these animals, and generally appear from 7-12 days after intracerebral infection. These workers have also demonstrated necrotic lesions in LCM carrier mice which had been reconstituted with LCM-immune spleen cells. These lesions were observed in the cerebellum, liver, and spleen and were generally unaccompanied by histologic evidence of inflammation. The significance of these lesions in terms of acute LCM virus disease has not been explored, but they do serve to illustrate a potential immunopathological genesis of necrotic disease. It does not appear, from the data obtained in the present study, that Pichinde virus disease in the adult MHA hamster represents an immunopathologic phenomenon. To draw this conclusion, however, will require further experimentation.

Persistent arenavirus infections can occur
in the presence of detectable antibodies to the virus;
the antibodies have been demonstrated by complement
fixation test in persistent LCM virus infection
(Hotchin, 1962; Oldstone and Dixon, 1969, 1970), Pichinde
virus infection (Trapido and Sanmartin, 1971) and
infections by other Tacaribe complex viruses (reviewed
by Johnson et al, 1973). The viral antigens against
which the antibodies are directed have not been established,
however from my results it would appear to be the soluble
CF antigen.

I found that the best antisera in terms of high
titer in CF test and sharp immunodiffusion reactivity
were convalescent sera from hamsters and guinea pigs
which had been infected with 2 X 10^4-10^6 PFU of virus
intraperitoneally. Although the inoculum used (stock
virus diluted 1:100-1:1000) contained between 0.02%
and 0.002% fetal calf serum, no antibody against FCS
proteins was detectable. The sera from both hamsters
and guinea pigs were also clean with respect to reaction
against non-infected BHK_{21} cells. The problem of
contaminating anti-BHK cell antibody activity was experienced
in attempts to raise rabbit antisera. Viremia was
demonstrated in both hamsters and guinea pigs after
virus inoculation. The anti-Pichinde virus antibody

produced was made in response to the infection in the
animal, and not in response to the relatively small
amount of virus in the inoculum. Replicating virus
appeared to supply a self-perpetuating antigenic
stimulus in the infected animal.

Convalescent sera apparently contained antibody
reactive to both soluble internal antigens and to
virus specific surface determinants of the infected cells.
Virus-neutralizing activity was not, however, detectable
in these sera. It cannot be stated at this time whether
or not the surface antigens detected on the infected
cells are the same as those present on the virion.
Furthermore, binding of antibody to the surface of the
virus may not result in neutralization of infectivity.
Reports by Webb et al (1970) and by Johnson et al (1973)
have described difficulties in neutralizing some of the
Tacaribe complex viruses. It is attractive to speculate
that this difficulty in neutralization is in some way
related to virus persistence. Neutralizing antibody
has been demonstrated in patients convalescing from both
Argentine and Bolivian hemorrhagic fevers (Teyssie et al,
1971; Webb et al, 1969), however, in Callomys callosus
infected as sucklings, virus persistence was never
accompanied by virus-neutralizing antibody (Justines and

Johnson, 1969). In C. callosus infected as adults,
two patterns were evident. Following an initial
viremia, some animals cleared virus from the blood and
developed neutralizing antibody, while in others the
virus persisted in the absence of neutralizing antibody
(Johnson et al, 1973).

The present studies have demonstrated two virus-
specific soluble antigens detectable by immunodiffusion
in saline extracts of cells infected with Pichinde virus.
Some of the demonstrated biophysical and biochemical
properties of these antigens are similar to those
described for the soluble antigens extracted from cells
infected with LCM virus (Bro-Jorgenson, 1971). Notable
among these is the ability to differentiate the two
antigens by the properties of heat stability and resistence
to proteolytic enzymes. In both the present study and
the previously reported study, one of the antigens was
resistent to heating and to pronase while the other
antigen was susceptible to both treatments. A similar
sedimentation constant of 3.5S was also found for both
of the antigens, and a diminution in the antigenic
activity of the sedimenting peak was observed if the
antigen preparation was heated prior to centrifugation.

An important difference noted in the data
presented here from that of the LCM study is in the
apparent molecular weight of the antigens. Bro-Jorgenson
estimated the molecular weight of the LCM antigen to be
48,000 based on an S value of 3.5. For the Pichinde virus
antigens I have determined an apparent molecular weight
of between 20,000 and 30,000 by gel filtration in two
different media. Analytical gel filtration in G-200
Sephadex, a dextran medium, yielded a value of
approximately 20,000 relative to marker proteins, while
subsequent experiments using Biogel P-200, a poly-
/acrylamide medium, have resulted in a molecular weight
estimate of 28,000. The difference in the two values
may reflect retardation of antigen elution in the
Sephadex gel, due to non-specific adsorption. This is
supported by the observation of a broader spread of the
eluting antigenic peak from the Sephadex column. In
the antigen peak eluting from both types of gel filtration
media, only the major (heat stable) antigen was detectable
by immunodiffusion. No other reproducible antigenic peaks
were demonstrable in Biogel P-200 or Sephadex G-200
eluates. Based on these observations, I have made the
assumption that the major antigen accounts for the bulk
of the antigenicity detectable by CF in the soluble

extracts of infected cells.

Isoelectric focusing of the major antigen in pH gradients was possible with high recovery of antigen. The major peak of antigenic activity, corresponding to the heat stable antigen, focused at pH 5.2. Analysis of the isoelectric focusing patterns of identical preparations from infected and control cells (Figure 21) revealed that there was no discernable peak of radio-labelled protein uniquely attributable to the focused antigen, suggesting that the CF antigen formed a quantitatively minor proportion of the total protein of the infected cells. This is not surprising in view of the non-cytocidal nature of the Pichinde virus infection in BHK_{21} cells. Neither Pichinde virus nor other arenaviruses shut off host macromolecular synthesis, and chronic infections in cell culture are easily established (Lehmann-Grube et al, 1969; Staneck et al, 1972; Rawls and McMillan, personal communication, 1975).

Partial purification of the major CF antigen from lysates of infected cells was difficult to accomplish. Although the antigen was readily detectable in such extracts by CF or immunodiffusion, and formed morphological inclusions which could be visualized in the cytoplasm of infected cells by immunofluorescence, the

fact that host-specific protein synthesis was not
suppressed compounded the difficulty of the purification
problem. Non-infected control cell extracts were
labelled and purified in parallel, and the products of
these purifications compared by acrylamide gel electro-
phoresis with the infected cell products. As illustrated
by the data in Table XI, the greatest increases in
specific activity of the purified antigen were obtained
by isoelectric focusing and subsequent gel filtration
of the focused antigen pool. Pooled antigen from an
isoelectric focusing gradient contained 6-7 major
polypeptides by SDS-PAGE. Of these, 2 appeared to be
specific for infected cells. Upon gel filtration of
the focused antigen all of the antigenic activity was
associated with the lowest molecular weight species
eluting from the column.

Further evidence of the purity of the eluted
antigen was shown by SDS-PAGE where polypeptides of
approximately 15,000 and 20,000 molecular weight were
observed and no demonstrable polypeptides were present
in the parallel control. The highest purification index
obtained was 40 fold over the starting 100,000 g super-
natant. In this experiment, approximately 25,000 CPM
of ^{3}H amino acid label was recovered from an initial

input of over 5×10^6 CPM. In terms of recovered CF antigen, the purification yielded approximately 3000 CF units of an initial 15,000 or 20% of the starting antigenicity. Assuming that multiple antigenic species were present in the original preparation, and that the quantitatively minor antigens resided in fractions other than those retained during purification of the major antigen, the recovery seems reasonably good.

The CF and immunodiffusion antigens have been shown to be viral components. This association is supported by the two lines of evidence presented in this study and by the work of others working with LCM virus (Gschwender, 1975; Gschwender and Lehmann-Grube, 1973). Initial evidence suggesting that the major antigen was virion associated came from experiments in which the NP-40 generated "core" components of fractionated virions induced, in immunized guinea pigs, antibody with specificity identical to the antiserum against major CF antigen derived from infected cells. The titer of the anti-core sera was comparable to that of antisera raised by inoculation with intact virus, and the reactions observed by immunofluorescence were similar for both the anti-viral and anti-core sera. One could argue that the anti-core serum was, in fact, an immune serum generated

by infection of the guinea pigs with a surviving
fraction of the original virus.   This possibility was
ruled out by demonstration of complete loss of
infectivity as judged both by plaque assay and by
failure to isolate virus from immunized guinea pigs at
6 and 9 days after inoculation; times when virus was
readily isolated from animals inoculated with intact
virus.   Since CF antibody was not induced after
inoculation with NP-40 solubilized virion components,
the antigen was tentatively associated with the core.

Further, more direct, evidence of the association
of the CF antigen with the virion came from experiments
in which milligram quantities of purified Pichinde
virus were treated with NP-40, ethyl ether and RNase
and then used as antigen in immunodiffusion.   As previously
shown (Figure 30, virus treated in this manner con-
tained antigens easily demonstrable by immunodiffusion.
The reason other workers have failed to demonstrate
the association of the CF antigen with the virion is two-
fold.   Most investigators (Smadel et al, 1939; Bro-
Jorgenson, 1971; Gschwender and Lehmann-Grube, 1973) have
attempted to detect CF activity in whole, undisrupted
virus.   It is evident from the data presented in this
thesis and from recent data presented by Gschwender (1975)
that the CF antigen is not represented on the surface of

the intact virion. Secondly, reproducible demonstration
of the antigen by immunodiffusion required, in my
hands, treatment of the NP-40 disrupted virus with
ribonuclease. This observation explained why previous
attempts to demonstrate antigenic activity by immuno-
diffusion virus had failed. This RNase mediated release
of antigen suggested that the antigen is associated in
some way with the virion RNA.

The association of RNA and the antigen within
the virus "core" was shown by another experiment.
Purified virus was treated with detergent then centrifuged
to pellet the "cores", and these were in turn treated
in RNase and again centrifuged on a 5-20% sucrose gradient.
Treatment of the previously insoluble "core" particles
with RNase released antigenic activity detectable by
CF and immunodiffusion which sedimented in the second
gradient at about 4S, demonstrating clearly that RNA was
necessary for the insolubility of the "core" antigens.
The structure of the putative "core" particle is un-
clear at this time. Typical cores have not been
demonstrated in ultrastructural studies of the arena-
viruses (Dalton et al, 1968; Murphy et al, 1970).
Given this lack of demonstrable core, the insoluble
material pelleted after NP-40 treatment may represent
a non-specific aggregation of virion proteins with RNA.

The polypeptide composition of this "core" particle was, however, repeatedly shown to consist of the V_I and V_{IV} polypeptides and the V_{II} glycopeptide. Antigen detectable by CF was clearly associated with the structure containing these polypeptides.

Gschwender (1975) working with LCM virus has demonstrated a similar association of antigen with virus. Using monospecific antiserum against the extractable CF antigen (ECFA) of LCM virus he has demonstrated activity by immunodiffusion against components of disrupted LCM virus. Antigenicity could not be assigned to either the surface of the virion or of the infected cell by several methods. In the present study we were unable to demonstrate reactivity of the antiserum against the major CF antigen at the surface of infected cells, although the cytoplasmic reaction was readily demonstrated.

The relationship of the two low molecular weight polypeptides observed in purified antigen with the "core" polypeptides was perplexing. In SDS-PAGE neither of the two polypeptides co-migrated with any of the "core" polypeptides, although the smaller of the two migrated close to V_{IV}, the smallest virion polypeptide. Based on these findings, one may propose two potential mechanisms to account for the relationship of the low molecular

weight polypeptides of purified antigen with the virion "core" polypeptides.

The first potential mechanism is that the smallest of the antigen polypeptides represents a modified form of the V_{IV} virion polypeptide. In the cell-associated form, the V_{IV} polypeptide may be slightly larger thus accounting for the small difference in electrophoretic mobility in PAGE. There is no direct evidence to support this hypothesis at the present.

Another potential mechanism for the generation of low molecular weight, antigenic polypeptides is by proteolytic cleavage of one of the larger virion polypeptides V_I or V_{II}. Cell associated V_I or V_{II} may be cleaved by proteolytic enzymes, present in the cytoplasmic extracts, yielding low molecular weight fragments of which one or both may retain antigenicity. That the antigenic fragment is resistant to proteolysis is supported circumstantially by the demonstration that the major antigen is pronase resistant.

Additional evidence to support the proteolytic cleavage hypothesis comes from data obtained by isoelectric focusing of the proteins of purified virus. In these experiments the major peak of CF antigen was always found associated with the larger polypeptides. This

suggested that the larger polypeptides carried the antigenic determinant.

Definitive proof of the molecular identity of the CF antigen awaits further study. An approach which I feel would be fruitful would be to label the viral polypeptides at high specific activity, purify each, then compare tryptic digest maps of these with maps of CF antigen purified from infected cells.

Pichinde virus has been previously shown to be inhibited by actinomycin D (Mifune et al, 1971), a characteristic shared with a number of other single stranded RNA containing viruses. Since actinomycin D inhibits the transcription of RNA from DNA, inhibition by this drug implies either RNA dependent-DNA polymerase activity (Temin, 1963) or a requirement during replication for host cell gene expression. Pichinde virus contains no detectable RNA dependent-DNA polymerase (Carter et al, 1974), therefore it was assumed that expression of host cell genes was necessary for replication. In the replication of influenza virus (Pons, 1973) early transcription of the viral RNA is blocked by actinomycin D. A similar blockage of viral RNA synthesis has been observed in the replication of lactic dehydrogenase virus (Brinton-Darnell et al, 1975) however, unlike

influenza virus, LDH virus yields were reduced when
the drug was added late in the replicative cycle
(Yamazaki and Notkins, 1975). The effect of actinomycin
D on Pichinde virus replication was similar to that
seen on LDH. Actinomycin D added late in the replicative
cycle was still inhibitory to the virus (Rawls et al,
1976). The present studies have demonstrated that
partial expression (antigen synthesis) of the Pichinde
virus genome occurs in the presence of actinomycin D
concentrations which are inhibitory to virus replication.
Normal, or nearly normal levels of antigen on a per cell
basis were synthesized, therefore appreciable translation
from the viral messenger RNA must have occurred. Since
the virion RNA does not appear to have a messenger
function (Dr. W. C. Leung, personal communication) this
implies prior transcription from the input virion RNA
or possibly a replicative intermediate.

There appears, on the basis of these data, to
be a requirement for host cell RNA synthesis in the
replicative cycle of Pichinde virus. The fact that all
of the identifable antigens were synthesized in the AD
treated, virus-infected cell coupled with the demonstrated
accumulation of viral antigen at the cell surface suggests
that the defect in replication in AD treated cells may
reside at the level of virus maturation. The lack of

detectable defective virus particles in the super-
natants of such cells along with no demonstrable increase
in cell-associated infectious virus lends credence to
this hypothesis.

A similar dissociation of virus replication from
antigen synthesis was observed in Vero cells infected
by Pichinde virus. In these experiments expression of
surface antigen and synthesis of new virus were found to
peak at 24-48 hr after infection then rapidly decline
thereafter. Internal antigen, on the other hand, re-
mained relatively constant for the duration of the 5 day
experiment. These data imply that some form of control
was imposed on the replication of the infecting virus.

Other investigators have noted dissociation
of antigen expression and virus synthesis in arenavirus
infections in vitro and in vivo. Lehmann-Grube et al
(1969) established a persistent infection in L cells with
LCM virus. Following repeated in vitro passage, virus
free clones of cells could be isolated which still
synthesized LCM virus-specific antigen detectable in the
cytoplasm by immunofluorescence.

Murphy et al (1976) have demonstrated antigen
synthesis in the absence of infectious virus in vivo.
In cotton rats persistently infected with Tamiami virus,

infectious virus disappeared from all organs tested
at around 40 days after infection. In the brain,
however, antigen detectable by immunofluorescence was
evident in the neurons for the duration of the 360 day
study.

These studies suggest that the arenaviruses
possess mechanisms by which virus production can be
shut off in the infected cell without complete loss
of viral genome expression. Whether this is accomplished
by a provirus-like mechanism similar to that seen in
SV-40 and polyoma transformation (reviewed by Butel
et al, 1972), and suggested for the oncornaviruses
(reviewed by Temin, 1971), or by some other as yet
undescribed mechanism is unknown. It is intriguing to
speculate that this sort of regulation of virus and
antigen expression plays an important role in the
establishment and maintenance of persistent infection,
but confirmation of this hypothesis awaits further
investigation.

SUMMARY AND CONCLUSION

While Pichinde virus has only been isolated from
Oryzomys albigularis under natural conditions, it appears
that the virus can infect a broad range of mammalian
hosts. Studies of the pathogenesis of Pichinde virus
infection in MHA and LVG hamsters have emphasized the
varied pathology of this arenavirus infection. By
manipulating virus-host combinations and age at the time
of inoculation, a range of pathological conditions from
inapparent infection (humans) to severe necrotic
disease (MHA hamsters) can be produced. Between these
extremes lies the previously documented persistent
infection (Trapido and Sanmartin, 1971).

Until this time, acute arenavirus disease in rodents was
thought to be primarily immunopathologic in nature.
The demonstration of Pichinde virus disease in MHA
hamsters, a condition apparently related directly to
virus replication, broadens the scope of arenavirus
induced disease. This model is interesting in that
disease ranging in severity from subacute to lethal
can be produced in adult animals of a single species;
the hamster. The studies of this model have demonstrated
significant differences between it and the other

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existing acute arenavirus diseases which have led to
the conclusion that immune responsiveness in the
hamster infected by Pichinde virus plays a protective
rather than detrimental role. Whether this model will
prove relevant to arenavirus disease in humans re-
mains to be seen.

The observation of persistence of LCM virus
in mice infected as newborns led Burnet and Fenner (1949)
to postulate that persistence was due to immunologic
tolerance to the virus. This characteristic of persistent
infection in animals infected early in life has been
demonstrated for nearly all members of the arenavirus
group. However it is now evident that the persistently
infected animals produce antibody to antigens induced by
the viruses. Tolerance as an explanation for viral
persistence can be questioned.

Based upon the results presented in this
thesis and upon the work of others, several possible
alternatives to absolute immunological tolerance can be
proposed. The first of these is a split type of tolerance
where an animal would respond with antibody formation to
one class of viral antigens (in this case the internal
CF antigens) but would not produce antibody capable of
neutralizing the virus. My data, which demonstrate
that CF antigen of Pichinde virus is an internal antigen

in the virion, is consistent with this concept. Antibody directed against the CF antigen has been demonstrated in persistent LCM virus infection (Oldstone and Dixon, 1969, 1970), Machupo virus infection (Johnson et al, 1973) and Pichinde virus infection (Trapido and Sanmartin, 1971) would not be expected to neutralize infectious virus. The fact that virus-neutralizing antibody is rarely, if ever, found in persistent arenavirus infections lends additional support to this hypothesis.

Failure of the cell-mediated arm of the immune response to function in combatting arenavirus infection could account for the generation of persistence. In this type of split tolerance, antibody production may function normally, but cell mediated immunity may be deficient. Support for this hypothesis is seen in the observation that resistence to lethal Pichinde virus infection in adult LVG hamsters can be overcome by treatment with cyclophosphamide, a procedure known to protect LCM virus-infected adult mice from immuno-pathologic death due to the cell mediated response generated in LCM virus infection (Gilden et al, 1972 a, b). In this model antibody production, and even

neutralizing antibody may be produced, but be ineffectual in overcoming the infection. Production of low levels of neutralizing antibody has recently been demonstrated in chronic LCM virus infection of the hamster (Parker et al, 1976), and antibody bound to virus in the blood of LCMV carrier mice was shown previously by Oldstone and Dixon (1969). The suppression of cell-mediated immunity may be the result of the previously demonstrated immunosuppressive effects of arenavirus infection (Bro-Jorgenson and Volkert, 1972a, b, 1974; Oldstone et al, 1973, Bro-Jorgenson et al, 1975). The immunosuppressive effects demonstrated appear to affect T-cell functions primarily, and in one report (Bro-Jorgenson et al, 1975) the effect is directed against cell mediated cytotoxicity functions.

An alternative to the split tolerance hypothesis is the idea that regulation of virus production and antigen synthesis in the infected cell may allow the virus to escape the host defenses. Several lines of evidence support the hypothesis that antigen expression and virus production are regulated in the persistently infected cell. Data presented in this thesis demonstrate that in infected Vero cells the production of new infectious

virus and the expression of surface antigen on infected cells shuts off between 48 and 96 hours after infection. Complement fixing antigen localized in the cytoplasm of the infected cells does not appear to shut off. Similar results have been reported in persistent LCM · virus infected cell cultures (Lehmann-Grube et al, 1969) and in chronic infection by Tamiami virus in cotton rats (Murphy et al, 1976). In both of these instances viral antigen was detectable in the cytoplasm of cells after the disappearance of infectious virus.

Factors external to the virus such as neutralizing antibody or interferon could conceivably affect this shutdown in vivo. Alternatively, factors related to virus replication, such as the generation of defective interferring (DI) particles, may be responsible. Such DI particles have been demonstrated in persistent LCM virus-infected cell cultures (Welsh and Pfau, 1972). The demonstration of a dissociation of antigen synthesis and virus replication in actinomycin D treated cell cultures has supplied a tool with which the intracellular events in replication may be approached. Through this sort of approach to the study of arenavirus replication I feel a better understanding of persistent arenavirus infections in vivo and in vitro will be gained.

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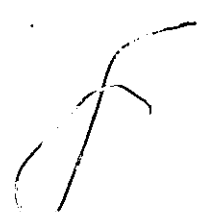
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APPENDIX I

February 11, 1976

Dr. Michael Buchmeier
Dr. William Rawls
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McMaster University-----
Hamilton,. Ontario L8S4J9
Canada

Dear Michael and Bill:

Our evaluation of the H & E slides of your study of Pichinde Virus Infection of Two Hamster Strains follows:

Newborn Hamster--8 Days Postinfection

Kidney: Extreme congestion and hemorrhage in cortex and medulla. Massive tubular necrosis, most severe at the corticomedullary junction (foamy to vacuolar necrosis). So many tubules are totally missing from the outer zone of the medulla that this zone appears rarified. It is clear that this appearance is mostly from loss of tubules and to a lesser extent, from edema. Necrotic debris is present in all interstitial spaces from the subcapsular space into the medulla. Karyorrhectic and pyknotic nuclei are present in many of the remaining tubules. Perhaps the most dramatic change concerns the presence of extraordinary numbers of cells within the lumen of the congested blood vessels and glomeruli. There also are fibrin-platelet thrombi. The inflammatory cells have very dense nuclei and narrow cytoplasmic rim. If all of these are blood monocytes (and few pmns), this would be the most fantastic concentration of such cells ever. These vessels may contain a mixture of such cells and karyorrhectic nuclei and debris from necrosis going on in other organs (such as lymphoreticular organs). These kidneys are incompatable with life.

Liver: Very large numbers of foci of necrotic hepatocytes are present throughout the liver specimens. The foci of cells which are totally destroyed are usually less than 10 cells in diameter, but a polychromasia of surrounding intact cells and nuclear changes in some of the same cells indicate that destruction of liver parenchyma is progressing much further than the edge of foci. In other words, there is the image of focal infection which has been bypassed by a confluent second phase of the infectious process. Some hepatocytes are sloughing into sinnsoids in intact condition. In some areas, it seems that there are too few Kupffer cells; at least it is clear that there is no proliferation or enlargement of these cells. This is contrasted by a moderate amount of monocytic inflammatory infiltration at or near sites of necrosis, near vascular triads and in sinusoidal lumina. The "focusing" of these cells at sites with the worst necrosis is not evident really. It also seems that there

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is too little phagocytic activity of any sort and an inordinate amount of
piling up of necrotic debris in the foci. Apparently, very little or none
of the karyorrhectic and eosinophilic debris is being carrier away. Coun-
cilman-like bodies are present. A few of the dense monocytic cells like
those seen in the kidney vessels are present in vascular bed of the liver.

Brain: Congestion is moderate. There is some mononuclear inflammation in
brain parenchyma and in brain surfaces (meninges). The striking lesions
are hemorrhages into the brain parenchyma from small vessels. The sites
of this hemorrhage are mostly in grey matter and at or near grey-white
matter junctions. They are distributed in olfactory bulb, cerebrum,
hippocampus and floor of lateral ventricle. In some sites, the vessel
wall damage may be seen--the karyorrhectic debris may reflect neuroglial
degeneration, inflammatory infiltration and endothelial necrosis. In
most sites, the RBC extravasation looks recent--cells still intact, but in
few cases, RBC breakdown is starting. One vessel in the cortex seemed
to be distended by a fibrin thrombus. Relative to the kidney and liver
lesions, this is much less significant--except for the importance of this
kind of small vessel hemorrhage as the analogue of human hemorrhagic fever.

Controls: Unremarkable

MHA Adult Hamsters

K1: Unremarkable

K8: Modest congestion particularly in the medulla. In one kidney, there
is very minimal tubular necrosis in foci located in the outer zone of the
medulla and not extending into the deep cortex. In the second kidney,
the same sort of tubular necrosis is present in one focus at the same
zonal location. There are also several small foci of necrotic tubular
epithelial cells in the deep cortex near the juxtamedullary capillary bed
source. The postglomerular capillary bed origin may be a place where
virus is most free to leave the bloodstream. Perihilar fatty tissue has
inflammatory (mononuclar infiltration but no other inflammation). Other-
wise, kidneys are normal.

K11: One kidney has an extremely large area of the outer zone of medulla
containing necrotic tubular epithelium--in this area, which includes
about ¼ of the area of the medullary outer zone, nearly all tubules are
involved. This necrosis is characterized by an apparently synchronous
destruction--tubule cells have pyknotic nuclei and cytoplasm in shreds.
No accumulation of the debris of this necrosis is present anywhere--no
cast formation. This tubular necrosis extends into the cortex but to a
lesser degree than in the medulla. There is modest interstitial capillary
congestion and glomerular congestion. The second kidney specimen is similar--
slightly less tubular necrosis and same perihilar infiltration as seen in
K8.

K15: There is some congestion of vessels in cortex, glomeruli and in the
outer zone of the medulla. There is minimal evidence of tubular necrosis--

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very few foci of pyknotic nuclei in the outer zone of the medulla. However,
in several foci in the cortex, large numbers of very deeply staining tub-
ular epithelial cells (with violet cytoplasm and black nucleus) are present.
These are necrotic, calcified epithelial cells--this identification is
confirmed at high magnification by the resolution of Ca apatite crystals
in some of these cells. In the absence of any inflammatory infiltration
into the kidney interstitium, this calcification represents an alternative
terminal pathway in the necrotic process. In some tubules, long rows of
these deeply staining cells are present, but in more sites, they occur as
individual or very small groups of cells intermixed with normal cells.
As judged by the number of nuclei per unit area of the tubule bed of the
outer zone of the medulla, it would seem that the amount of tubular re-
generation was very large at this time. The second kidney specimen is
identical, except that no active tubular necrosis is evident. Overall,
the magnitude of the regenerative process is such that the kidney disease
is not likely to be the immediately lethal event--of course, animals which
had died earlier may have presented a downhill progression from the day
11 stage, in which case this organ would be considered a major target.
Such is the problem with a model with a ragged death curve--what is the
difference between individual animals which die first, last, and those
which survive? The lack of evidence of RBC extravasation or RBC presence
in the lumen of intact or necrotic tubules fails to correlate with the
hematuria which is seen in these animals--there is no histologic evidence
of any hemorrhagic manifestation in these kidneys, but because of the loss
of so many tubular epithelial cells, there should be a fantastic protein
content in urine. The survival of the basement structures of tubules is
consistent with the precision of the regenerative process.

K19: No congestion, no necrosis, no calcification. As at day 15, the
tubules in the outer zone of the medulla appear to have more nuclei (a
higher concentration of cells) than the K1 specimens (=control). This
would be evidence of tubule regeneration; the other evidence of tubular
epithelial regeneration, namely the scalloped luminal profile of this
epithelium, was not seen.

L1: Unremarkable. The glycogen content of these liver specimens is
very high--the normal baseline must include this index of the superior
nutritional condition of these animals.

L8: Large numbers of foci of necrotic hepatocytes involving from 10 to
50 cells per focus in the plane of section. There are several hundred of
these foci in the plane of section; about 10 percent of the liver parenchyma
is affected. Focus distribution is random in lobules. There is some poly-
chromasia of surrounding hepatocytes (eosinophilia) which is probably
indicative of progressive necrosis. There is also considerable evidence of
cell division, indicative of regeneration beginnings. There are few hepatocytes

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with extremely basophilic cytoplasm--necrosis with calcification beginning.
As in the liver specimens of the newborn hamster, there is no evidence
of any proliferation or swelling of Kupffer cells, and in areas near necrotic
foci there seem to be fewer Kupffer cells than normal. Few inflammatory
cells are associated with the necrotic foci (monocytes in sinusoids and
near vascular triads) than in the newborn hamsters. Necrotic debris
remains in situ (karyorrhectic nuclei and eosinophilic cytoplasmic debris).
Councilman-like bodies present. No sign of abnormal extravasation of
blood cells and no congestion.

L11: The two specimens differ only in that one has marked fatty change;
both have massive number of foci of necrotic hepatocytes. These foci
are randomly distributed in lobules--in virtually every lobule. The
size of foci is somewhat larger than in L8--up to 100 cells per focus in
the plane of section. There is no apparent Kupffer cell proliferation
and no phagocytic removal of necrotic debris--as in L8, all of the debris
appears to remain in situ. The amount of monocytic infiltration of
perivascular and parenchyma areas seems too modest--perhaps too diffuse
to see. Moderate number of mitoses and binucleate cells. No congestion
and no RBC extravasation. Overall, the amount of damage is striking,
and although the proportion of the parenchyma reduced to debris is about
10 percent, cells that are likely in earlier stages of the necrotic
progression may constitute another 10 percent. This magnitude of acute
liver necrosis may or may not be considered potentially lethal--but in
relation to the other observations, this hepatitis must be considered the
central pathologic event (of course, lymphoreticular organs not examined
yet). A question regarding these liver specimens is the nature of materials
within sinusoids. In areas of necrosis, one consideration was that this
debris was largely made up of materials from another target organ (namely
lymphoreticular system) which had been carried to liver sinusoids in the
blood. However, this is not the case--this debris seems clearly to be
the product of destruction of the hepatocytes at the same site where the
debris piles up. Again, very few RBCs are present in abnormal sites, and
there is no evidence of hemorrhage.

L19: One liver specimen is normal with a high concentration of glycogen.
The second liver specimen is the same except that few individual hepato-
cytes and even fewer small groups of cells are basophilic (magenta cytoplasm)--
at high magnification these cells may be seen to be undergoing necrosis with
calcification. No inflammatory cells are associated with these dark hepato-
cytes. Kupffer cell population is normal.

B1: Unremarkable

B8: Unremarkable

B11: Brain surfaces normal except for one small focus of inflammatory in-
filtration into meninges (round cells). Brain parenchyma normal.

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B15: Brain surfaces normal. Brain parenchyma normal except for a necrotic focus in the posterior commisure of the hippocampus. This lesion had indistinct margins and consisted of a rather basophilic debris and lucent space filling its center. This lesion is next to a small vessel. There are no inflammatory cells in or near this lesion. In general, this lesion looks like the bland necrotic foci which occur in the brain of mice infected with LCM or Tacaribe viruses or LCM in rats. There are few other foci which possibly are similar in nature but are too indefinite to be sure. Another lesion with more of the lucent, bubbly space and less basophilic interstitium is present in the putamen.

B19: No necrotic foci seen and all brain surfaces normal. A moderate amount of perivascular cuffing (round cells) is primarily located in areas adjacent to the floor of the brain (midbrain). These cuffs surround medium sized vessels and are two or three cells thick. No neuronophagia.

LVG Hamsters

K1: Unremarkable

K8: Modest amount of tubular necrosis focally present in the outer zone of the medulla (same site as in MHA)--evidenced by pyknotic nuclei and dissolution of tubule cells. No casts. Modest degree of congestion and inflammatory infiltration of hilar fatty tissue (no other inflammation).

K11: A definite progression of the tubular necrosis is evident--more cells per tubule, and more tubules contain necrotic cells. Little necrosis of cells in deep cortex; center of necrotic foci still in outer zone of medulla. Overall, the foci of tubules with necrotic cells are in only one quadrant of the medulla of each kidney--and even in this quadrant less than half of the tubules are involved, and a minority of cells in affected tubules are necrosed. No inflammation, no congestion, no hemorrhage, no cast formation.

K15: The amount of tubular necrosis is similar to that on day 8 and less than on day 11. In each specimen, there are one or two large foci in the outer zone of the medulla, but in each focus, few tubules and few cells are involved. Typically, cells with pyknotic nuclei are intermixed with normal cells--probably indicating regeneration. No inflammation, no hemorrhage or congestion, no cast formation and no calcification.

L1: Unremarkable

L8: One liver specimen normal with high glycogen content. The second liver specimen, despite similar high glycogen content, has a modest number of foci of necrotic hepatocytes. These are randomly distributed in lobules. There are about 30-40 foci in the plane of section. Foci vary from 1 to 30 cells

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in the plane of section. The foci are different than those in MHA hamsters: the hepatocytes are nearly intact, with pyknotic nuclei and smooth or granular dark cytoplasm (Magenta). Where cell margins are lost, the center of foci have a confluent granular appearance. The character of the cytoplamic debris in these sites suggests early calcification. Kupffer cell population appears normal, even near the necrotic foci. There is none of the karyorrhectic debris as found in focal lesions in MHA hamsters. Absolutely no inflammation. This is an extremely bland necrosis.

L11: Both liver specimens are identical to the L8 specimen with necrotic foci--about 20 small foci per liver profile are present, and these are the same small size as in L8. The same bland necrosis without nucleorrhexis leaves calcifying cells nearly intact in the lesion site without any in-flammation.

No L15 specimen.

No lesions found in any brain specimen of the LVG series.

Conclusions: Fantastic set of slides and the most beautiful H & E stain we have seen in a long time.

1. Expand organ and tissue collection in next run--especially to include lymphoreticular organs (thumus, LN, spleen and marrow). Include salivary gland (submandibular) and as many other organs as you can manage. Include cerebellum (may separate from rest of brain). Age matched controls needed.

2. Do send conjugate and dilution data with the frozen tissue.

3. Max Chernesky, Virology Lab, St. Joseph's Hospital in Hamilton is interested in platelets vs. viruses--he would be interested in collaborating with you, I believe, since arenaviruses really hit megakaryocytes.

4. Karl Johnson, who now has labs across the hall, was very interested in your observations. With full credit to "you all," he is ordering some MHA hamsters to infect with Lassa virus. We are also very interested in your observations with peritoneal wash cells--fantastic virus yield--on a "per cell" basis, it must be as high or higher yield as ever gotten with an arenavirus (like the human lymphoblastoid cell-Machupo virus pair in our J. Virology paper years ago). How about some EM of macrophages at peak yield time?

5. It is too early to draw conclusions, but...

a. There is no histologic hemorrhagic disease in MHA adults to mimic human disease--if hematuria is real, it must be very subtle histologically.

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b. However, the liver disease (focal necrosis) is very much like the human Lassa fever we have seen—a range of necrotic changes from bland to vicious and too little host reactivity. This may be the real merit of your model.

c. The newborns have a very interesting disease which may be most valuable in comparison with MHA vs. LVG adults.

d. The LVG animals are great for our microscopic comparisons (as well as your quantal comparisons)—we were surprised at the magnitude of the necro.sis—although the difference in the two strains is great enough to explain life vs. death. The blandness of the necrosis in LVG was striking—like petrification.

e. All in all, is it possible to suspect that the key to the outcome of these infections concerns the relation of virus with macrophages? The only exception to this would be in the LVG adults where liver (and kidney) lesions were resolved without any cellular influx.

f. <u>Many</u> slides forthcoming (as soon as developed and labeled). — *included*

We will be in touch by phone soon.

Sincerely yours,

Fred

Frederick A. Murphy, D.V.M., Ph.D.
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