IMMUNOLOGIC STUDIES ON PICHINDE VIRUS
IMMUNOLOGIC STUDIES ON PICHINDE VIRUS INFECTION IN CELL CULTURE AND IN SYRIAN HAMSTERS

By

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A Thesis
Submitted to the School of Graduate Studies
in Partial Fulfilment of the Requirements
for the Degree
Doctor of Philosophy

McMaster University
June 1976
DOCTOR OF PHILOSOPHY (1976)  McMaster UNIVERSITY
(Medical Sciences)  Hamilton, Ontario

TITLE: Immunologic Studies on Pichinde Virus Infection
in Cell Culture and in Syrian Hamsters

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NUMBER OF PAGES: xvii, 210
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 complement-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 resistant protein of 20-30,000 molecular weight estimated by gel filtration. The minor antigen was heat labile and susceptible to proteolysis. Antiserum prepared against partially purified major antigen, yielded a band of identity when tested against antiserum
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, isolectric 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 interfering 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 BHK₂₁ cells is blocked at the level of virus maturation.
ACKNOWLEDGEMENTS

I would like to thank Dr. Rawls for support, guidance, and encouragement in these studies, and the entire Rawls family, for friendship both in and out of the lab. Thanks are also extended to Ms. Carol McMillan, Ms. Elaine Jaggard and Dr. Rama Rao Gangavalli for assistance in the performance of some of the experiments described. The contribution of Dr. Jack Gauldie in supplying helpful discussion and suggestions regarding protein purification is greatly appreciated.

I would also like to thank Dr. Frederick Murphy, in Atlanta, for evaluating the histopathology of Pichinde virus infection in hamsters, and Ms. Terry DeCola for preparing this manuscript.

Very special thanks are offered to Nancy and to Josh for love, patience, understanding and moral support.

This work described in this study was supported by grants from the National Cancer Institute of Canada, and the Medical Research Council.
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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 arenavirus 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
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 characteristics 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 serologically 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 hyperimmune Lassa antiserum 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**

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<td>60-280 nm</td>
<td>LCMV</td>
<td>Dalton <em>et al.</em>, 1969</td>
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<td></td>
<td>Machupo</td>
<td>Abelson <em>et al.</em>, 1969</td>
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<tr>
<td></td>
<td></td>
<td>Murphy <em>et al.</em>, 1969</td>
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<tr>
<td>50-150 nm</td>
<td>Amapari, Latino</td>
<td>Murphy <em>et al.</em>, 1970</td>
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<td>Tacaribe, Tamiami</td>
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<td></td>
<td>Parana, Pichinde</td>
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<tr>
<td></td>
<td>Lassa</td>
<td>Speir <em>et al.</em>, 1970</td>
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<td></td>
<td>Junin</td>
<td>Lascano and Berria, 1969</td>
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<td><strong>Sedimentation Rate in Sucrose Gradients</strong></td>
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<tr>
<td>470-500S</td>
<td>LCMV</td>
<td>Pederson, 1970</td>
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<tr>
<td>300-325S</td>
<td>Pichinde</td>
<td>Ramos <em>et al.</em>, 1972</td>
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<tr>
<td>76S</td>
<td>LCMV</td>
<td>Pfau, 1965</td>
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<td><strong>Bouyant Density in Sucrose</strong></td>
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<tr>
<td>1.18 gm/cm$^3$</td>
<td>Pichinde</td>
<td>Mifune <em>et al.</em>, 1971</td>
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<td></td>
<td>Amapari</td>
<td>Johnson <em>et al.</em>, 1973</td>
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<td></td>
<td>LCMV</td>
<td>Pederson, 1973a</td>
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<tr>
<td>1.17 gm/cm$^3$</td>
<td>Junin, Machupo</td>
<td>Johnson <em>et al.</em>, 1973</td>
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<td></td>
<td>Tacaribe</td>
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<tr>
<td>1.12 gm/cm$^3$</td>
<td>Junin</td>
<td>Coto <em>et al.</em>, 1972</td>
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<td><strong>Bouyant Density in Amidotriazoate</strong></td>
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<td>1.14 gm/cm$^3$</td>
<td>LCMV</td>
<td>Gschwender <em>et al.</em>, 1975b</td>
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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 biophysical and biochemical properties of these structures isolated from Pichinde virus. The particles display sedimentation and bouyant density properties similar to BHK₂₁ 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 4-5S
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 sedimentation 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 bromodeoxyuridine (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-azaauridine 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 involved 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 glycoproteins, 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 $V_{III}$ represented 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. Smedel 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 BHK₂¹ 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 (1938). 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 glomerulonephritis 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 (Sigmodon hispidus) 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 Oryzomys albicularis (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 O. albicularis. Of 220 O. albicularis animals tested, 43 (20%) were positive for virus whereas only 1 of 988 animals of 15 species other than O. albicularis was positive for Pichinde virus. Similar host-virus relationships exist for other Tacaribe group arenaviruses (reviewed by Johnson et al, 1973).
VI. Acute Disease Following Arenavirus Infection

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, *Mus musculus*. 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 primarily 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) demonstrated that pre-treatment of mice with immunosuppressive 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 antiviral 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 as 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 pathogenicity 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 immunopathological 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 decreased 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 neonatally 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 lipopolysaccharide, pneumococcal polysaccharide 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 $5 \times 10^6$ 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. Purpose of the Study

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 in vivo.

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 unraveling 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. Cell Culture and Virus Growth

A. Cell Culture

BHK$_2$ and Vero cell lines were grown in stationary or rolling monolayer cultures in plastic flasks (Corning). Cells were grown to confluence using Eagle's minimal essential medium (MEM) supplemented 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 streptomycin, 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°C. Medium was changed after the first 24 hr of incubation, and cells reached confluence 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°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°C in an atmosphere of 5% CO₂.

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 BHK₂₁ 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 BHK₂₁ 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 concentrations 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 remaining 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 $2.5 \times 10^4$ 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 $-50^\circ C$ 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 supernatant 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

FREEZE THAW TWICE

SONICATE - 3 1 MIN CYCLES

CENTRIFUGE 60 MIN, 100,000 × g

SUPERNATANT ➔ PELLET ➔ DISCARD

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

ISOELECTRIC FOCUS ON pH 3.5 - 10 GRADIENT - POOL FRACTIONS CONTAINING HIGHEST ANTIGEN CONCENTRATION

DIALYSE AGAINST PBS

EMULSIFY IN COMPLETE FREUND'S ADJUVANT

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

GUINEA PIG ANTISERUM AGAINST MAJOR (CF) ANTIGEN

**Figure 1:** Flow chart of methods used to obtain antiserum against partially purified Pichinide 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°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 CH₅₀ 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% NaN₃. 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 immunodiffusion 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 coverslips. 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°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°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 monolayers on coverslips was done by a previously described method (Rutter and Gschwender, 1973). Briefly, monolayers 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 trypsinized 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 X 10⁵ 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 125I IgG binding to viable, suspended cells. Hamster IgG was purified by 3 precipitations with saturated (NH₄)₂SO₄ 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 125I. 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 10^5 cells per tube. The cells were pelleted at 1000 RPM for 3 min, the supernatant drained from the tubes, and 50 ul of 125I 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 radioactivity 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 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® 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. Density Gradient Centrifugation

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 125I 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®, LKB). Two percent Ampholyne® 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% H₂SO₄ 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 μg/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\textsuperscript{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, \textsuperscript{3}H-counts per minute were corrected for spillover from the \textsuperscript{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\textdegree 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 fluoroautography method of Bonner and Laskey (1969). Exposures of dried, PPO impregnated gels were carried out at -70\textdegree C for 7-14 days to detect \textsuperscript{3}H and 4-7 days to detect \textsuperscript{14}C. Films were processed in a Kodak X-omat\textsuperscript{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 interfering components in the sample (i.e. electro-focusing 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 (ProcysntoR, 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 mortar 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°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 quantities of purified Pichinde virus into rabbits. Antiserum 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
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 (○○) and control (△△) BHK$_{21}$ cells are shown.
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 hyperimmune 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 SUSPENSION IN ADJUVANT

<table>
<thead>
<tr>
<th>Source (Pool of)</th>
<th>CF Reactivity</th>
<th>ID Bands</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Hamster</strong> (Pool of 12)</td>
<td>Virus-Specific: 1:2048, Anticompl. 3: 1:32</td>
<td>+(1:4)</td>
</tr>
<tr>
<td><strong>Mouse</strong> (Pool of 30)</td>
<td>1:160, Anticompl. 3: 1:4</td>
<td>+(1:8), +(1:1)</td>
</tr>
</tbody>
</table>

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 $2.5 \times 10^4$ 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.
Figure 4

Immunodiffusion reaction between serum from Pichinde virus-immune guinea pigs and soluble antigen prepared from virus-infected and control BHK₂₁ 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 BHK₂₁ 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.
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 resistant 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 resistant 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 observed daily for death.
Figure 6

Mortality among adult MHA hamsters after intraperitoneal infection with various doses of Pichinde virus. Virus doses used were: (○○) \(3.5 \times 10^6\) PFU; (△△) \(3.5 \times 10^4\) 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

<table>
<thead>
<tr>
<th>Age at Inoculation (days)</th>
<th>Virus titer$^1$</th>
<th>Day 4</th>
<th>Day 9</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Kidney</td>
<td>Brain</td>
</tr>
<tr>
<td>3</td>
<td>6.2</td>
<td>3.3</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>4.9</td>
<td>&lt;1</td>
<td></td>
</tr>
</tbody>
</table>

$^1 \log_{10}$ virus titer in PFU/gram.

$^2$ Day after inoculation.
TABLE IV

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

<table>
<thead>
<tr>
<th>Age at Inoculation</th>
<th>Day 4</th>
<th>Day 9</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 days</td>
<td>5.8</td>
<td>8.4</td>
</tr>
<tr>
<td>6 days</td>
<td>3.5</td>
<td>5.3</td>
</tr>
<tr>
<td>12 days</td>
<td>3.2</td>
<td>4.1</td>
</tr>
<tr>
<td>Adult (&gt;30 days)</td>
<td>3.0</td>
<td>&lt;3.0</td>
</tr>
</tbody>
</table>

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 infected with $2 \times 10^5$ PFU of Pichinde virus intraperitoneally, and two animals were tested at each time indicated.
<table>
<thead>
<tr>
<th>Organ</th>
<th>Hamster</th>
<th>Day After Inoculation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Strain</td>
<td>1</td>
</tr>
<tr>
<td>Blood</td>
<td>MHA</td>
<td>0</td>
</tr>
<tr>
<td>(serum)</td>
<td>LVG</td>
<td>0</td>
</tr>
<tr>
<td>Kidney</td>
<td>MHA</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>LVG</td>
<td>0</td>
</tr>
<tr>
<td>Spleen</td>
<td>MHA</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>LVG</td>
<td>0</td>
</tr>
<tr>
<td>Brain</td>
<td>MHA</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>LVG</td>
<td>0</td>
</tr>
</tbody>
</table>

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.
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; (○○○○○) MHA macrophages.
macrophages of both strains of hamsters supported virus
growth equally well.

4. The Effect of Cyclophosphamide Immunosuppression
   on Resistance to Infection

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. Anti-Viral Antibodies in LVG and MHA Hamsters
   after Pichinde Virus Infection

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 immunosuppression on mortality in adult LVG and MHA hamsters. The animals were infected with $2.5 \times 10^5$ 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 (○○○) 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

<table>
<thead>
<tr>
<th>Hamster Strain</th>
<th>Day After Infection</th>
<th>CF Antibody Titer&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Virus Specific&lt;sup&gt;2&lt;/sup&gt;</th>
<th>Anticomplementary&lt;sup&gt;3&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>LVG</td>
<td>10-13 (3)&lt;sup&gt;4&lt;/sup&gt;</td>
<td>1:53&lt;sup&gt;5&lt;/sup&gt;</td>
<td>≤1:2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>21 (2)</td>
<td>1:256</td>
<td>≤1:2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>30 (5)</td>
<td>1:128</td>
<td>≤1:2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>60 (7)</td>
<td>1:64</td>
<td>≤1:2</td>
<td></td>
</tr>
<tr>
<td>MHA</td>
<td>10-13 (5)</td>
<td>1:38</td>
<td>≤1:2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>21 (2)</td>
<td>1:128</td>
<td>1:4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>30 (4)</td>
<td>1:128</td>
<td>1:21</td>
<td></td>
</tr>
</tbody>
</table>

<sup>1</sup> Highest dilution giving 50% endpoint in complement fixation test against 5 units of antigen.

<sup>2</sup> Determined against antigen prepared from Pichinde virus infected BHK<sub>21</sub> cells.

<sup>3</sup> Fixation of complement in the absence of virus specific antigen.

<sup>4</sup> Number of samples tested is indicated in parentheses.

<sup>5</sup> 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 $2 \times 10^5$ 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 $2.5 \times 10^5$ 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 removed from Pichinde virus infected and control newborn
TABLE VII

MORTALITY AMONG VARIOUS HAMSTER STRAINS AFTER PICHINDE VIRUS INFECTION

<table>
<thead>
<tr>
<th>Hamster Strains</th>
<th>Survivors¹ Initial Number</th>
<th>Percent Mortality</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Exp. 1</td>
<td>Exp. 2</td>
</tr>
<tr>
<td>LVG</td>
<td>20/23</td>
<td>-</td>
</tr>
<tr>
<td>MHA</td>
<td>5/20</td>
<td>-</td>
</tr>
<tr>
<td>LHC</td>
<td>6/6</td>
<td>10/11</td>
</tr>
<tr>
<td>PD-4</td>
<td>6/6</td>
<td>5/5</td>
</tr>
<tr>
<td>CB</td>
<td>2/6</td>
<td>4/4</td>
</tr>
<tr>
<td>LSH</td>
<td>3/3</td>
<td>-</td>
</tr>
</tbody>
</table>

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 $2 \times 10^5$ PFU of virus intraperitoneally. All tissues were placed immediately into 10% buffered formalin and histologic sections were prepared and stained with hematoxylin 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 (45%) 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

<table>
<thead>
<tr>
<th>Risk Group</th>
<th>No. Tested</th>
<th>CF Antibodies to Pichinde virus</th>
<th>No. Positive</th>
<th>% Positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>High</td>
<td>13</td>
<td></td>
<td>6</td>
<td>46</td>
</tr>
<tr>
<td>Moderate</td>
<td>14</td>
<td></td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Low</td>
<td>17(^1)</td>
<td></td>
<td>0</td>
<td>0</td>
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</tbody>
</table>

\(^1\) 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 injecting 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 fourfold 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.
GF ANTIBODY TITER

DATES OF BLEEDINGS


MC  BR  GH  FF  MB  WR
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 intra-
cellular 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 cytopatho-
genic effect which could be detected in viable preparations
or in Giemsa stained specimens. The total numbers of
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 BHK₂₁ 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 μg/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>
<thead>
<tr>
<th>Actinomycin D Concentration (µg/ml)</th>
<th>Virus Titer</th>
<th>Cell Associated CF Titer</th>
<th>Antigen Units/mg</th>
<th>Protein %</th>
<th>Virus Yield</th>
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<tbody>
<tr>
<td>0</td>
<td>8 x 10^7</td>
<td>100</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1</td>
<td>4 x 10^6</td>
<td>7</td>
<td>4.9</td>
<td>66.5</td>
<td>4.5</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>4 x 10^5</td>
<td>0.5</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

1. Virus titer in supernatant fluids measured 48 hr after infection.
2. Cell associated antigen detected in cell lysates by complement fixation test 48 hr after infection.
3. Percentage of cells infected determined by direct immunofluorescent staining of acetone fixed cells.
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 cytoplasmic 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
Immunofluorescent staining of the surface of cells infected with Pichinde virus. BHK21 cells were infected with Pichinde virus, incubated 24 hr. at 37°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 BHK₂₁ cells were grown in 75 cm² 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 ³H-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 actinomycin 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 (○—○). Uninfected cells, no actinomycin D (▲—▲); uninfected cells and actinomycin 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, V1, 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 BHK21 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 interfering (DI) particles. To examine this, supernatant fluids from AD-treated cultures
Virus-specific polypeptides present in BHK\textsubscript{21}
cells infected with Pichinde virus and incubated
with or without actinomycin D. Six 75 cm\textsuperscript{2} flasks
of BHK\textsubscript{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 \textmu g/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 \textmu Ci/ml \textsuperscript{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 fluororautoradiography. The lanes
contained the following (1) \textsuperscript{3}H glucosamine labelled
Pichinde virus marker (80,000 CPM). (2 and 9) \textsuperscript{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.
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\textsubscript{21} cell monolayers in two 75 cm\textsuperscript{2} flasks were infected with 1-3 PFU/cell of Pichinde virus. After adsorption 1 \mu g/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 \textsuperscript{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 continuous gradient. Fractions from the gradients 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 (\textsuperscript{-}\textsuperscript{-}\textsuperscript{-}); infectious virus, actinomycin D (\textsuperscript{+}\textsuperscript{+}\textsuperscript{+}); \textsuperscript{3}H-amino acids, no actinomycin D (\textsuperscript{0}\textsuperscript{--}O); \textsuperscript{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<sub>21</sub> 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°C. One antigen was relatively stable at 56°C while the other antigen could no longer be detected by immunodiffusion after 20 minutes at 56°C. When soluble lysates were exposed to 25 μg/ml of pronase for 60 min at 37°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 μg/ml of ribonuclease-a for 60 min at 37°C. By autoradiography of dried immuno-
Figure 18

Immunodiffusion test showing the effect of heating on the antigens detected in lysates of BHK21 cells infected with Pichinde virus. The perimeter wells contained soluble antigen which was heated at 56°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>
<thead>
<tr>
<th>Treatment</th>
<th>CF Titer</th>
<th>Immunodiffusion Band Observed</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>/ Major</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>128</td>
<td>+²</td>
</tr>
<tr>
<td>Incubation at 22°C 24 hr</td>
<td>128</td>
<td>+</td>
</tr>
<tr>
<td>Incubation at 56°C 60 min</td>
<td>86</td>
<td>+</td>
</tr>
<tr>
<td>Digestion with 25 ug/ml RNase at 37°C 60 min</td>
<td>128</td>
<td>+</td>
</tr>
<tr>
<td>(pH 7.2)</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>Digestion with 25 ug/ml pronase at 37°C 60 min</td>
<td>ND</td>
<td>+</td>
</tr>
<tr>
<td>(pH 7.2)</td>
<td></td>
<td>-</td>
</tr>
</tbody>
</table>

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 \(^{14}\text{C}\) 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 \(2 \times 10^4\). 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 NaN₃) and standardized using the indicated marker proteins. A lysate of virus-infected BHK₂₁ cells was clarified by centrifugation at 100,000 x g for 60 min, and 1.2 ml of the supernatant was applied to the column. Fractions of 2 ml were collected and assayed for antigen by CF.
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 BHK21 Cells

Taking advantage of the characteristics described above, a scheme was derived to partially purify the major CF antigen from lysates of infected BHK21 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 (4.1S), 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 isoelectric focusing gradients and focused for 72 hr at 300 v. Fractions were collected and the radioactivity 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 antigenicity 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

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

1. Protein content determined by the method of Lowry et al. (1951).
2. CH50 antigen units in test with 4 units of immune hamster serum.
3. Total CF antigen units / Total mg protein
4. Specific activity of 12,000 x g supernatant
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 preparations 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 (○○) 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 indirect 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 45 min

CENTRIFUGE 150,000 g

90 min

3.5 x 10^10 PFU/ml

CFA

GUINEA PIGS

IMMUNE SERUM

SUPERNATANT 0 PFU/ml

CFA

GUINEA PIGS

ANTI VIRION SURFACE

PELLET 0 PFU/ml

CFA

GUINEA PIGS

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 antisera against NP-40 obtained virus "cores" (ANTI CORE) and partially purified CF antigen (ANTI AG).
CELL LYS

ANTI AG

ANTIGEN

CORTEX

IMMUNE
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 Richinde 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.
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

<table>
<thead>
<tr>
<th>Immunogen</th>
<th>Animal No.</th>
<th>CF Antibody Titer</th>
<th>Antibody Reaction</th>
<th>Immunodiffusion</th>
<th>Immunofluorescence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact virus</td>
<td>1</td>
<td>1:64</td>
<td>2 Bands</td>
<td>2 Bands</td>
<td>Positive granular cytoplasmic staining and reticular surface reaction.</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1:128</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NP-40 disrupted virus - Insoluble core fraction</td>
<td>1</td>
<td>1:32</td>
<td>1 Band (major)</td>
<td>1 Band (major)</td>
<td>Positive granular cytoplasmic staining Negative surface.</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1:64</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NP-40 disrupted virus 1 solubilized fraction</td>
<td></td>
<td>&lt;1:2</td>
<td>Negative</td>
<td></td>
<td>Positive vacuolar cytoplasmic staining</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1:8</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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\textsubscript{21} cells infected with Pichininde virus.
4 Indirect immunofluorescent staining of Vero cells infected with Pichininde virus.
Immunodiffusion reaction showing identity of antigens detected in lysates of infected BHK₂₁ cells (CELL LYS) with antigens detectable in disrupted Pichinde virus (DIS V). Sera used were Pichinde-immune guinea pig serum (IMM S) and monospecific 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

<table>
<thead>
<tr>
<th>Gradient Fraction</th>
<th>Antigenicity¹</th>
<th>Protein</th>
<th>RNA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Major</td>
<td>Minor</td>
<td>CPM² % of Total</td>
</tr>
<tr>
<td>Soluble material</td>
<td>+⁴</td>
<td>+</td>
<td>1780</td>
</tr>
<tr>
<td>P&lt; 1.05</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Middle fractions</td>
<td>-</td>
<td>-</td>
<td>nil⁵</td>
</tr>
<tr>
<td>P= 1.16 - 1.18</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pelleted fraction</td>
<td>+</td>
<td>+</td>
<td>690</td>
</tr>
<tr>
<td>P= &gt;1.24</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1 Antigen bands detected by immunodiffusion.
2 CPM³H amino acid label/50 μl aliquot.
3 CPM¹⁴C 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 antigenicity 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-
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.
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 µg/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 (○○○).
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 $^3$H amino acids, was purified as described above (Table XI) then concentrated by 15% TCA precipitation and compared with viral polypeptides by SDS-polyacrylamide gel electrophoresis. Comparisons were made by coelectrophoresis of $^3$H-amino acid labelled antigen with $^{14}$C amino acid labelled polypeptides 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 (―——―).
TABLE XIV

RELATIVE DISTRIBUTION OF THE FOUR MAJOR VIRION STRUCTURAL POLYPEPTIDES AFTER NP-40 SOLUBILIZATION AND RNase DIGESTION

<table>
<thead>
<tr>
<th>Treatment of purified virus</th>
<th>$V_I$</th>
<th>$V_{II}$</th>
<th>$V_{III}$</th>
<th>$V_{IV}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>71%</td>
<td>10.4%</td>
<td>13.8%</td>
<td>4.8%</td>
</tr>
<tr>
<td>NP-40 Solubilization</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Soluble (72%)$^2$</td>
<td>70%</td>
<td>8.4%</td>
<td>16.7%</td>
<td>5.3%</td>
</tr>
<tr>
<td>Insoluble &quot;Core&quot; (28%)</td>
<td>46%</td>
<td>39.5%</td>
<td>0</td>
<td>14.4%</td>
</tr>
<tr>
<td>RNase treated core run on 5-20% sucrose gradient</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4S fract. (85%)</td>
<td>16%</td>
<td>60%</td>
<td>0</td>
<td>24%</td>
</tr>
<tr>
<td>Pellet (15%)</td>
<td>92%</td>
<td>8%</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Immune precipitin of 4S fract. (96%)$^3$</td>
<td>18%</td>
<td>62%</td>
<td>0</td>
<td>19.6%</td>
</tr>
</tbody>
</table>

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
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 VII 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,
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 precipitated 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.
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; (○—○) $^{14}$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,
fractons 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 radiolabeled 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 visibly 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 \( V_I \)
polypeptide 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 μg/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 (---).
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.
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 albicularis* (Trapidio 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 (Trapidio 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. albicularis* rodents. These mites did not survive well in the laboratory, so that long term observation was impossible. These workers
could not eliminate the possibility that the virus isolated reflected surviving virus in engorged viremic blood.

Humans are susceptible to a number of arenaviruses 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 concentrated 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
contagious 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 immunosuppression, 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; Trapiño and Sanmartín, 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 resistance 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 immunosuppression 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 resistance 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 of virus doses, from 35 to $3.5 \times 10^6$ 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 severely 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 immunosuppressant 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 resistant 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 noticeably 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 noticeably 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 LYG 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 × 10⁴-10⁶ 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<sub>21</sub> 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 intracellular 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-Jørgensen, 1971). Notable among these is the ability to differentiate the two antigens by the properties of heat stability and resistance to proteolytic enzymes. In both the present study and the previously reported study, one of the antigens was resistant 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 polyacrylamide 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
isolectric 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 $5 \times 10^6$ 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 pig 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 contained antigens easily demonstrable by immunodiffusion. The reason other workers have failed to demonstrate the association of the CF antigen with the virion is twofold. Most investigators (Smadel et al., 1939; Bro-Jorgenson, 1971; Gschwender and Lehmann-Grube, 1973) have attempted to detect CF activity in whole, undischrupted 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 immunodiffusion 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 unclear at this time. Typical cores have not been demonstrated in ultrastructural studies of the arenaviruses (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.

Gechwender (1975) working with LCM virus has demonstrated a similar association of antigen with virus. Using monospecific antiserum against the extractable CF antigen (LCFA) 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 than 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 $V_1$ or $V_{II}$. Cell-associated $V_1$ 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 identifiable 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 supernatants 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, remained 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.
While Pichinde virus has only been isolated from *Oryzomys albicularis* 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
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 remains 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 Sanmartín, 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 resistance 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 immunopathologic 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., 1975), 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 interfering (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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APPENDIX I
February 11, 1976

Dr. Michael Buchmeier  
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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 incompatible 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 sinusooids 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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Dr. Randles

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 carried away. Councilman-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 juxtedudillary 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 (mononuclear infiltration but no other inflammation). Otherwise, 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—
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 tubular 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 regeneration 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 polychromasia 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 hepatocytes 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 hepatocytes. Kupffer cell population is normal.

B1: Unremarkable

B8: Unremarkable

B11: Brain surfaces normal except for one small focus of inflammatory infiltration 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 commissure 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 cytoplasmic 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 necrosis leaves calcifying cells nearly intact in the lesion site without any inflammation.

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 (thymus, 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.
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. LVC adults.

d. The LVC animals are great for our microscopic comparisons (as well as your quantal comparisons)—we were surprised at the magnitude of the necrosis—although the difference in the two strains is great enough to explain life vs. death. The blandness of the necrosis in LVC 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 LVC adults where liver (and kidney) lesions were resolved without any cellular influx.

f. Many slides forthcoming (as soon as developed and labeled). **included**

We will be in touch by phone soon.

Sincerely yours,

Fred

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