THE ROLE OF *CELLULAR AND* HUMORAL IMMUNITY IN THE PATHOGENESIS OF EXPERIMENTAL PlCHINDE VIRUS INFECTION IN MICE.

BY:

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ABSTRACT

The role of cell mediated and humoral immune response in the pathogenesis of experimental pichinde virus infection in CBA mice was investigated. The study showed, that cell mediated immunity as measured by invitro mediated lysis of ^{51}cr-labelled target T lymphocyte L cells played a vital role in the pathogenesis of acute lethal pichinde virus infection of suckling CBA nice. During the period of maximum lysis (8-9 days post infection) clinical symptoms were apparent in the acutely infected mice, at the sane time histopathological changes consisting of inflammatory cell infiltrations and necrosis of vital, organs such as the liver, kidneys and brain were prominent removal of T lymphocytes by antithymocyte serum resulted in considerable reduction of mortality while depletion oi macrophages or B lymphocytes did not significantly reduce mortality or cytotoxicity. Adoptive transfer of immune lynphocytes, bes obtained from syngeneic pichinde virus infected mice to persistent pichinde virus carrier mice also resulted in mortality. While humoral immunity did not contribute significantly to the pathogenesis of acute pichinde virus infection of suckling CBA mice neither did antibody dependent cell mediated cytotoxicity play a significant role in the pathogenesis of the infection. It was observed that in persistently infected pichinde virus carrier CBA mice, antibody played a vital role in the pathogenesis of pichinde virus infection as these persistent pichinde virus carrier mice

developed circulating immune complexes which gradually deposited in the kidneys of the carrier mice and contributed to glomerulonephritis, fibrinoid necrosis and perivascular inflammations which resulted ultimately to the death of most of the carrier mice. Removal of virus -antivirus immune complexes from the sera of these carrier nice by precipitation with anti mouse gamma globulin resulted in significant reduction of infectivity.

Although it was shown in this dy that H-2 identity between the effector and target cells would yield maximum lysis of pichincle virus infected target cells, there was no significant difference in susceptibility of the four inbred strains of mice ering in H-2 haplotype to acute pichinde virus infection is all the inbred strains were equally susceptible to lethal pichind e virus infection.

This study also revealed that mononuclear cells from CBA mice either acutely or persistently infected with pichinde virus yield infectious virus when measured by infectious centre assay though the yield from acutely infected mice was higher than the yield from persistent carriers, furthermore mitogenic stimulation of these cells gave increased levels of infectious centre forma-tion which might suggest that these cells might serve as reservoir for virus persistency.

Striking differences between the immune phenomena, pathology and clinical manifestations of pichinde virus infection and the type or model arenavirus disease lymphocytic choriomeningitis (LCM) were observed. The expirations, which may be in age, strain and aex-dependent regulatory factors , may be very important in finding a Key to prophylaxis or therapy in the arenavirus - caused human diseases which include the type disease LCM and also Lassa fever, endemic in Nigeria. Future studies will be oriented in this direction.

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D E C L A R A T I O N

This thesis by Aliyu Ahmad meets the regulations governing the degree of Doctor of Philosophy of Ahmadu Bello University and is approved for its contribution to Scientific knowledge and literary presentation.

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TABLE OF CONTENTS

Chapter I	Page
GENERAL INTRODUCTION	1
The Virus	1
General Characteristics of Arenaviruses	2
Biochemical and Biophysical Characteristics	3
Effect of Arenaviruses on the Immune Function	5
Immune Response to Arenavirus Infections	8
Deleterious Effect of Immune Response	8
Effect of Humoral Immunity on Pathogenesis	11
Role of Immune Complexes in Arenavirus Pathogenesis	14
Genetic Control of Host Susceptibility to Arenavirus Infection	16
REVIEW ON PICHINDE VIRUS	19
Historical background	19
Biochemical and Biophysical Properties of Pichinde Virus	19
Pichinde Virus Proteins	21
Antigenic Properties of Pichinde Virus	21
Pathogenesis and Immunity to Pichinde Virus	22
Immune Response to Pichinde Virus Infection	23
Transmission of Pichinde Virus	25

Chapter 2. Page

MATERIALS AND METHODS 27
Viruses 27
Cell lines 27
Mice 28
Sera 28
Isotopes 29
Reagents 29
Plates & Tubes 30
Other reagents 30
Medium for virus growth 30
Medium for virus plaque assay 31
Agar overlay 31
Tryptose phosphate broth 31
Stock balanced salt solution 31
Glycine-HCl buffer 32
10% neutral buffered formalin 32
Phosphate buffered saline 33
Veronal buffer stock solution 33
Alsevors solution 33
Buffers for C1q Isolation and Testing 34
Solutions for C1q Isolation 34
Solutions for C1q binding test 35
Polyethylene glycol solution 35
Borate buffer 35
Veronal Buffered Saline 35
Ethylene diamine tetra acetic acid buffer ... 35

Chapter 2 (Continued) Page

Alkali Aggregate 36

Michaelis buffer 36

METHODS USED IN THIS STUDY 36

Preparation of Virus Stock 36

Preparation of anti Pichinde Immune ascitic fluid 37

Preparation of anti pichinde antibody in rabbits 38

Extraction of complement fixing antigen from infected cell lines 38

Complement fixing antigen from organs of infected mice 38

Virus titration in mice 39

Complement fixation test 39

Plaque assay 40

Preparation of Antithymocyte serum 41

Fractionation of lymphocytes by Nylon Wool Column 42

Preparation of Nylon Wool Column 42

Loading and separation of cells 42

Lymphoid cell preparation using commercial ficollpaque 43

Macrophage depletion 44

T-cell depletion 44

B-cell depletion 45

Cytotoxicity assay 45

Effect of Immune spleen cells on the course of Pichinde virus Infection 46

Antibody mediated cytotoxicity 47

Effect of Cyclophosphamide 48

Chapter 2 (Continued) Page

Histology 48

Infectious Centre Assay 49

Infectivity of sera from pichinde carrier mice 49

Determination of Pichinde virus - gammaglobulin complexes in blood of pichinde carrier mice 50

Procedure for staining tissues with H&E 50

PAS staining 51

Effect of Histocompatability difference on Cytotoxicity 51

Virus titre and mean survival time 52

Preparation and Isolation of Clq..... 53

Isolation of Clq 53

Purity control 54

Method for Radio-Iodination of Clq 55

Solution for labelling 55

Labelling Clq 56

Assessment of uptake 56

Clq binding test 56

Chapter 3. Page

RESULTS 58

Age related susceptibility to lethal pichinde virus infection 58

Effect of virus dose on lethality 60

The Effect of cyclophosphamide on Pichinde virus Infection 62

Role of cell mediated Immune response in the pathogenesis of acute Pichinde virus infection in CBA mice 68

Virus concentration in organs of acutely infected mice 70

Histopathology of acutely infected CBA mice. 77

Histopathology of persistently infected carrier mice 94

Infectious Centre activity of Acutely infected and Persistent carrier mice 95

Effect of T-cell depletion 96

Effect of macrophage depletion on cytotoxicity of virus infected targets 100

Blastogenic response of lymphocytes from infected mice 103

Effect of histocompatability difference on cytotoxicity 105

Mean survival time 107

Effect of T-cells depletion 107

Role of Humoral Immunity on the Pathogenesis of experimental Pichinde Virus Infection of Mice. 110

INTRODUCTION 110

Effect of humoral immunity on pathogenesis of Pichinde virus 113

Chapter 3 (Continued) Page

Effect of antithymocyte serum on the course of pichinde virus infection 116

Effect of Adoptive Transfer of Immune Spleen Cells to Persistent Pichinde Carrier CBA Mice. 122

The role of Immune complexes in the pathogenesis of Pichinde virus in CBA mice 124

Chapter 4 Page

DISCUSSION 129

Cell mediated immunity in the pathogenesis of Pirchinde virus infection 129

Histocompatability Difference 133

Immune Complexes In Pathogenesis of Pichinde Virus. 134

LIST OF TABLES

Table		Page
3.1:	Effect of mouse age and route of inoculation on susceptibility to lethal death due to pichinde virus infection in CB1 mice	59
3.2:	Effect of Pichinde virus dose on lethality to suckling CB1 mice: Intracerebral inoculation.	61
3.3:	Effect of Cyclophosphamide on the course of pichinde virus infection.	64
3.4:	Infectivity titre of cyclophosphamide treated and acutely infected mice with pichinde virus...	65
3.5:	Infectivity titre of CB1 mice acutely infected with pichinde virus.....	71
3.6:	Cytotoxicity assay in Suckling mice acutely infected with pichinde virus.....	72
3.7:	Cytotoxicity assay in pichinde infected Immune mice.	73
3.8:	Infectious centre activity in mononuclear cells of CB1 mice acutely infected or persistent infected with Pichinde virus	97
3.9:	Effect of T-cell depletion on cytotoxic effect of pichinde virus infected CB1 spleen cells on target L-cells	99
3.10:	The effect of Macrophage depletion on Specific Cytotoxicity.	101
3.11:	Effect of treatment with rabbit antimouse Immunoglobulin plus Complement on Cytotoxicity.	102
3.12:	Effect of mitogenic stimulation of lymphocytes on infectious centre formation	104
3.13:	Cytotoxic effects of spleen cells from different strains of mice acutely infected with pichinde virus	106
3.14:	Effect of Histocompatability difference on susceptibility to acute pichinde virus infection	108

LIST OF TABLES CONT'D.

Table		Page
3.15:	Effect of T-cell depletion on cytotoxicity of spleen cells from different inbred mouse strains to pichinde infected Cr-51 labelled target L-cells	109
3.16:	Complement fixing and neutralizing antibody titres in acutely infected weanling CBA mice.....	115
3.17:	Effect of Antithymocyte Serum on Mortality of Pichinde Infected CBA Mice.	117
3.18:	Complement fixing antibody titre and viremia in Persistently infected pichinde virus carrier CBA mice	119
3.19:	Infectivity of serum from carrier mice persistently infected with pichinde virus	120
3.20:	Effect of Treatment of Pichinde carrier CBA serum with Antimouse gamma globulin on infectivity.....	124
3.21:	Effect of Adoptive transfer of Immune Syngeneic Spleen cells to persistently Infected pichinde Carrier CBA Mice	123

LIST OF FIGURES

Figure		Page
3.1:	Effect of immune spleen to target cell ratio on ^{51}Cr. released from pichinde virus infected target L cells	68
3.2.	Sequential development of cytotoxic activity in spleen cells of CBA mice acutely infected with pichinde virus	69
3.3a,b:	Log virus titre in the brain and liver of mice acutely infected with pichinde virus....	74
3.3c,d:	Log virus titre in the spleen and blood of mice acutely infected with pichinde virus....	75
3.4a,b:	Log virus titre in the kidneys and brain of mice acutely infected with pichinde virus	76
3.5:	Infectious centre activity of mononuclear cells of CBA mice acutely infected and persistently infected with pichinde virus..	98
3.6:	Specific ^{51}Cr. released from pichinde virus infected cells by anti pichinde serum plus complement	112
3.7:	Calibration curve of C1q binding assay.....	125
3.8:	C1q binding activity in sera of individual CBA mice persistently infected with pichinde virus compared to those age matched uninfected controls	126
3.9:	C1q binding activity in sera of individual mice persistently infected with Pichinde virus	127
3.10:	Mean serum Immunoglobulin levels of persistent pichinde carrier mice (open bars) compared to uninfected control mice of the same age group (matched bars).	128

LIST OF PLATES

Plate		Page
3.1a:	Photomicrograph of liver of mouse infected with Pichinde virus. Note the lymphocytic infiltration around the bile duct. H & E stain x 400	79
3.1b:	Liver of mouse infected with Pichinde virus showing mononuclear cellular infiltration	80
3.2a:	Photomicrograph of liver of mouse infected with Pichinde virus. Note the swobllen hepatocyte (giant cell) and kupffer cells (black arrow). H & E. stain x 400	81
3.2:	Photomicrograph of spleen of mouse infected with Pichinde virus. Note necrosis of the white pulp and giant cells. H & E stain x 100	82
3.3a:	Photomicrograph of kidney of mouse infected with Pichinde virus. Note coagulation necrosis of the convoluted tubules. H & E stain x 100.	83
3.3b:	Photomicrograph of kidney of mouse infected with Pichinde virus. Note coagulation necrosis of the convoluted tubulets. H & E stain x 400	84
3.4a:	Photomicrograph of brain of mouse infected with Pichinde virus. The choroidal epithelium was edematous and infiltrated with mononuclear cells. H & E stain x 400 ...	85
3.4b:	Photomicrograph of brain of mouse infected with Pichinde virus. Note perivascular cuffing with mononuclear cells (top left corner) and choroid epithelium infiltrated with some mononuclear cells. (top). H & E stain x 400	86
3.4c:	Photomicrograph of brain of mouse infected with Pichinde virus. Showing marked astrocytic and microglial reaction H & E stain x 100 ...	87

LIST OF PLATES (CONT'D)

Plate		Page
3.5.	Photomicrograph of lung of mouse infected with Pichinde virus. Note septal thickening resulting in narrowing of the alveolar lumens..	88
3.6a:	Photomicrograph of liver of mouse infected with Pichinde virus. Note some pyknotic nuclei around the central vein. H & E stain x 100.....	89
3.6b:	Photomicrograph of liver of mouse infected with Pichinde virus. Note hepatocyted with swollen nuclei and which had undergone hydropic degeneration. H & E stain x 400	90
3.7a:	Photomicrograph of kidney of mouse infected with Pichinde virus showing perivascular cuffing with lymphocytic cells. H & E stain x 100	91
3.7b:	Photomicrograph of kidney of mouse infected with Pichinde virus showing perivascular cuffing with lymphocytic cells. Note the lymphocytes are found and near the glomeruli too. H & E stain x 100	92
3.7c:	Photomicrograph of kidney of mouse infected with Pichinde virus showing perivascular cuffing with lymphocytic cells. H & E stain x 400	93

C H A P T E R O N E

1.1.0. GENERAL INTRODUCTION

1.1.1. The Virus:

Pichinde virus is a member of the Tacaribe complex of arenavirus taxonomic group which include lymphocytic choriomeningitis virus (the prototype virus), lassa virus (the agent of lassa fever), and the tacaribe complex of viruses (Junin-the etiologic agent of Argentinian haemorrhagic fever, Machupo-the etiologic agent of Bolivian haemorrhagic fever, amapari, tamiami, tacaribe, latino and parana viruses). All except lassa and lymphocytic choriomeningitis viruses are found localized in South and Central America. Lassa virus is so far found in West Africa only. LCM virus is widely spread. Lassa, Junin, Machupo and to a lesser extent LCM viruses are the only known human pathogens among the arenaviruses so far discovered. Lymphocytic choriomeningitis virus was the first of the arenaviruses to be discovered from human autopsy materials of fatal case suspected to be Saint Louis Encephalitis virus (Armstrong and Lillie, 1934). The virus was also isolated from patients with aseptic meningitis (Rivers and Scott, 1936). In this group of viruses, the next member to be described was Tacaribe virus which was isolated from bats in Trinidad in 1956 (Downs et al., 1963). This followed the isolation of amapari virus (Pinheiro et al., 1966), Junin virus from a patient with Argentinian haemorrhagic fever syndrome (Parodi et al., 1966),

Machupo virus from fatal cases of Bolivian haemorrhagic fever (Johnson et al.,1965b and Webb et al., 1967), Latino and parana viruses (Webb et al., 1970, Tamiami virus (Calisher et al., 1970), lassa virus (Buckley and Casals, 1970 and Spier et al., 1970). Pichinde virus was first isolated in 1965 from pooled liver, spleen and kidney tissues obtained from a rodent Oryzomys albigularis which was trapped in a forest in the pichinde valley of Colombia South America but was not characterized until 1971 (Trapido and San Martin, 1971).

1.1.2. General Characteristics of Arenaviruses:

The arenaviruses have all negatively stranded RNA genome. They are grouped together on the basis of similarities observed in the virus structure, RNA content and serological relationship among the members of the group. Electron microscopic examination of arenavirus infected cells or negatively stained virus preparation have revealed that the virus particles are spherical or pleomorphic measuring (50-300nm in diameter) with 10nm club shaped projections (Dalton et al., 1968, Abelson et al., 1969, Murphy et al., 1969, 1970, 1973, Buckley and Casals 1970, Mifune et al., 1971 and Ofodile et al., 1973).

Examination of ultrathin sections of all arenaviruses has shown that the viruses characteristically possess within their virions one or more 20-24nm diameter electron

dense granules which have been shown to be 80s ribosomes of host cell origin (Carter et al., 1973; Pedersen, 1973b; Farber and Rawls, 1975). It was from the sandy appearance of the ribosomal granules that the name arenavirus was derived.

1.1.3. Biochemical and Biophysical Characteristics:

Biochemical and Biophysical studies have shown that all arenaviruses appear to be similar although important differences do exist. All the viruses possess single stranded RNA in four large pieces and several smaller pieces with a total molecular weight of approximately 3.5×10^6. The RNA species have sedimentation co-efficients of 31S, 28S, 22S, 18S, 15S, and 4-6S. The 31S, 22S and 15S are virus specific while the other RNA species are of host ribosome origin (Pedersen 1970, 1971, 1973, Carter et al., 1973; Carter, 1973; Pfau, 1974; Pfau et al., 1974; Farber and Rawls, 1975 and Anon et al., 1976). The virus specific RNA species are negatively stranded as they lack the structural and functional properties associated with positive stranded mRNA such as polyadenylated sequences at the 3' termini and the RNAs are noninfectious per se (Carter et al., 1973, 1974; and Leung and Rawls, 1977.

The arenaviruses have four major structural polypeptides two of which are glycosylated and the others nonglycosylated (Ramos et al., 1972), Segovia and

Demitri (1977). Purified antigens derived from arenavirus infected cells have been individually examined. Soluble complement fixing antigen derived from Guinea pig tissues infected with LCM virus was studied and found to be separable from the virus by ultra centrifugation (Smadel, 1939). Bro-Jorgenson (1971) detected two antigenic species in extract of LCM virus infected BHK-21 cells using immunodiffusion technics. One antigen was heat stable and resistant to pronase digestion while the other antigen was heat labile and sensitive to pronase degestion, but both antigens sedimented at approximately 3.5S in sucrose gradients. A similar result was obtained by Buchmeier et al., (1977) when studying pichinde virus infected cells. The heat stable antigen, when purified by sucrose gradient centrifugation followed by isoelectric focusing and gelfiltration, was found to account for a major portion of the complement fixing activity. The soluble complement fixing antigen was distinct antigenically from the virion as inoculation of complement fixing antigen into animals induced antibodies but failed to neutralize the virion (Gschwender and Lehmann-Grube, 1973). On the basis of this complement fixing activity and indirect immunofluorescence staining the relationship between individual members of the arenaviruses was established (Mettler et al., 1963; Casals, 1965 and Rowe et al., 1970). The glycosylated antigen which is externally located and is associated with the envelope

of the virus is specific for each arenavirus species and is used in neutralization test for type specific identification of the arenaviruses.

1.1.4: Effect of arenaviruses on the Immune Function:

It is well-established that certain viruses can infect and subsequently alter the morphology of the lymphoid organs. Electron microscopic studies have demonstrated the presence of virus particles in cells of the lymphoreticular system such as macrophages, lymphocytes, thymocytes etc. Murphy et al (1976) reported the infection of megakaryocytes by taniami virus and in a similar study pichinde virus particles were demonstrated in macrophages of infected mice (Murphy et al., 1977).

Lymphocytic choriomeningitis virus was also found to persistently infect macrophages and both T&B lymphocytes (Popescu et al., 1979). These authors hypothesized that the lymphocytes in the LCM virus carrier mice were infected via the LCM virus specific antigen receptors thereby leading to their antigen triggered clonal expansion followed by infection and functional inactivation. Some viruses can replicate in lymphoid cells and destroy the cells although not all infections of the immune system results in cell destruction, some may lead to persistent infections. For example infection of B lymphocytes by Epstein-Barr virus can result in the establishment of a continuous lymphoid cell line in vitro, while infection

with leukemia viruses may result in malignant transformation.

It is generally believed, however that resting murine lymphocytes are not permissive to infection by viruses unless the cells have undergone blast transformation following exposure to a specific antigen or mitogen (Eustatia and Van der Veen, 1971, Kano et al., 1973).

Replication of viruses in lymphocytes or macrophages can affect the function of the immune system. Investigations by Mims and Wainwright (1968) have shown that mice infected with lymphocytic chorioneningitis virus made four to eight fold less antibody to sheep red blood cells than did the uninfected controls. A similar study (Parodi et al., 1967) had earlier shown that infection of guinea pigs with Junin virus resulted in the decrease of both the primary and secondary immune response to human red blood cells.

Other viruses were found to enhance the immune response instead of depressing it. Notkins et al., 1 (1966) and Howard et al., (1969) showed that infection of mice with lactic dehydrogenase virus (LDV) or Venezuelan Equine Encephalitis virus (VEE) can enhance the immune response to certain antigens.

In addition to their effect on humoral immune response, virus infection can also affect the cellular immune response. It has been shown (Hanaoka et al., 1969) that infection of mice with LCM virus resulted in the destruction of thymic dependent areas of the spleen and lymph nodes. Allograft rejection was profoundly depressed in animals infected with gross Leukemia virus (Dent, 1972). Infection with rubella and measles viruses have been shown to inhibit blast transformation of lymphocytes (Olson et al., 1968). Willems et al., (1969) showed that lymphocytes from patients with infectious mononucleosis and infectious hepatitis have depressed ability to undergo blast transformation.

The consequences of viral depression of immune response could make the host more susceptible to other infections. Depression of cellular immunity could make the host less able to reject malignant cells which could enhance growth of tumours. It could also account for chronic nature of certain virus infections (Rawls, 1968; Cihak and Lehmann-grube, 1974, and 1978). Virus enhancement of immune response is believed to be important in the etiology of autoimmune diseases by stimulating auto antibodies.

Oldstone (1970) showed the prevalence of auto-immune diseases in NZB mice which had enhanced immune response.

1.1.5. Immune Response to Arenavirus Infections:

Immune response to infectious agents is an important component of the host defense mechanisms. It is generally agreed that specific immune response against viral infections plays a decisive role in the recovery from these infections (Glasgow, 1970 and Blanden, 1971). In certain viral infections humoral immune response plays an important protective role while in other infections recovery from primary infection and resistance to reinfection is mediated by cellular immune response that involves specifically sensitized thymus derived 'T' lymphocytes (Henney, 1973 and Cerottini and Brunner, 1974). T-cell mediated lysis of cells expressing viral antigens may be generally beneficial in cases where sensitized T-cells act either alone to eliminate virus transformed tumour cells as shown in rejection of allogeneic tumours (Freedman et al., 1972) or T-cells can collaborate with macrophages to limit the growth of cytotoxic viruses in tissues (Blanden, and Mims., 1973)

1.1.6. Deleterious Effect of Immune Response:

In a number of cases the immune response of the host may be the principal cause of the pathological manifestation of certain viral infections. Evidence that T-cells operate to the detriment of the host has been shown in a number of lympho-proliferative diseases such as Marek's disease of chicken (Rouse et al., 1973), the post infectious viral encephalitis and various persistent

viral infections (Hotchin, 1971).

In arenavirus infections of mice the immune response of the host has been found to play a key role in the pathogenesis of the infection especially when damaged cells are essential for the functional intergrity of the host. This has been intensively investigated with lymphocytic choriomeningitis virus the prototype of the arenaviruses because LCM virus produces life long persistent infection in new born mice while mice infected as adults develop a fatal immunopathologic disease of the central nervous system. LCM virus infection in adult mice is not cytopathic and the animal remains free of clinical signs until the onset of the immune response about a week after intracerebral infection when inflammatory cell infiltrations occuring at visceral and meningeal sites of virus replication initiate the pathological changes leading to death of the animal. Immunopathological nature of the disease was proved by showing that inhibiting the development of cell mediated immune response by neonatal thymectomy or administration of antilymphocyte serum or cytotoxic drugs such as cyclophosphamide would prevent the lethal disease, (Rowe et al., 1963; Cole et al., 1971; Gilden et al 1972). Doherty and Zinkernagel, 1974; Johnson and Cole, 1975.

In contrast to the acute lethal disease produced in adult mice neonatally or congenitally infected mice develop no clinical signs of the disease during the early

phase of the infection eventhough widspread infection of the tissues have been demonstrated, but later in life these carrier mice develop chronic glomerulonephritis as a result of deposition of virus antibody complexes in the kidneys of these mice (oldstone and Dixon 1969, 1970, 1971).

Attempts have been made to determine whether it is the antibody mediated or cell mediated component of the immune response that causes the pathological manifestations of the disease. Invitro experiments by Lundstedt 1969; Oldstone and Dixon, 1970; Marker and Volkert, 1973; Doherty and Zinkernagel, . 1974 have all shown that spleen cells from LCM virus immune donors destroy LCM virus infected syngeneic target cells. The peak cytotoxicity of lymphoid cells from the infected mice co-incided with the time the pathological manifestation of the acute disease. The specific cytotoxicity was abrogated by prior treatment of these cells with anti theta ascitic fluid plus complement but was unaffected by treatment with anti mouse immunoglobulin serum plus complement; neither would depletion of macrophages have significant effect on cytotoxic activity of the immune spleen cells. This strongly indicated that thymus derived T-cells were responsible for the pathologic manifestation of the acute disease. These T-cells were specific as they did not exhibit cytotoxic activity for monolayers infected with other viruses (Doherty, Zinkernagel and Ramshaw,

1974). The result agreed with data obtained with *in vivo* experiments by Cole *et al* (1971), Gilden *et al* (1972) who showed that when LCM virus immune spleen cells were transplanted into cyclophosphamide induced persistent carriers, the mice died with the acute pathological manifestations similar to those occuring in natural acute infection. These results clearly indicate that cell mediated immune response plays a vital role in the pathogenicity of LCM virus.

Further studies by Volkert *et al* (1974) indicated the presence of two subsets of T lymphocytes in LCM virus infected host. An early short lived T cells were entirely responsible for cytotoxic activity against LCM virus infected target cells. Antiviral effect after transfer to acutely infected animals was also the function of the early immune T-cells. These cells were inactive after transfer to chronically infected virus carrier mice whereas transplant of late immune T-cells (Longlived) to such mice had very strong antiviral effect. The early immune T-cells were found to be resistant to X-irradiation whereas late immune cells were sensitive. Both were however susceptible to anti theta serum treatment.

1.1.7. Effect of Humoral Immunity on Pathogenesis:

Studies by Oldstone and Dixon 1971 have shown that LCM virus antibody plus complement is cytotoxic for LCM virus infected target cells *in vitro* as the absence of

specific antibody and complement resulted in no injury. The possible immunopathological action of antibody was also suggested by finding that complement depletion made LCM virus considerably less lethal for adult mice (Oldstone and Dixon, 1971).

In many viral systems it has been shown that virus infection can produce new antigens on the surfaces of infected cells. The interaction of specific antiviral antibody and complement with the surface antigens can lead to cell destruction and may contribute to the pathogenesis of the lesions associated with the infection. Experimental evidence in vivo with LCM virus has demonstrated that antibody can play pathogenetic role when passively administered to animals infected with LCM virus as intensification of characteristic lesions resulted (Oldstone and Dixon 1969, 1971, 1972).

The degree of injury depends on the density of viral antigen on the surface of infected cells, the inherent susceptibility of the cells to lysis by complement, the nature and concentration of the antiviral antibody, the ratio of complement fixing to non complement fixing antibodies in the serum and the presence of inhibitors such as rheumatoid factor that might block the complement fixing sites on the antiviral antibody.

Recently Johnson et al., 1978 have demonstrated that B-cells and their secreted products did not play a vital role in the pathogenesis of acute central nervous system disease caused by LCM virus. Other studies by Welsh et al., 1977; Welsh (1978) have demonstrated the involvement of natural killer cells (NK) in the pathogenesis of acute LCM virus infection.

Much of the evidence regarding the pathogenesis of arenavirus infections has been derived from studies of LCM virus in mice; the pathogenesis of other arenaviral infections has received little attention. The pathogenesis of Tacaribe virus in mice was found (Borden et al., 1971) to be immune mediated as they demonstrated that the acute lethal effect of the virus could be prevented by prior treatment with anti-lymphocyte serum which also prevented the development of the histopathologic lesions. Immune ascitic fluid failed to influence the course of the disease indicating that the disease was primarily mediated by cellular component of the immune response.

It was also shown that pathogenicity of Junin virus was immune mediated as it was prevented by neonatal thymectomy (Weisenbacher et al., 1969). The pathogenicity of Amapari virus was however found not to be immune mediated as thymectomy or treatment with antilymphocyte serum did not protect the mice from the lethal effect of the virus (Parodi et al., 1967). Murphy et al., 1977 studied the course of Pichinde virus infection in

hamsters and concluded that the lesions were due to direct viral cytopathogenic effects rather than immunopathologic host response as found in other arenaviruses.

Among the arenaviruses only Junin, Machupo and Lassa viruses have been proven to be human pathogens (Johnson et al., 1965; Justines and Johnson, 1969; and Buckley and Casals, 1970). These viruses are extremely hazardous and only laboratories with maximum containment facilities have been able to study the viruses. Therefore very little is known about their mechanisms of pathogenicity.

Differences in pathogenicity do exist in some of the arenaviruses while pathogenicity of Tacaribe complex of viruses decrease with increase in mouse age at infection, the pathogenicity of the prototype LCM virus increases with increase in age at inoculation (Johnson et al., 1973 and Borden et al., 1974). This work will try to investigate the role of cellular and humoral immunity in confering resistance or in the pathogenesis of pichinde virus infection in inbred strain of CBA mice.

1.1.8 Role of Immune Complexes in Arenavirus Pathogenesis:

It is well established that the persistence of antigen-antibody complexes in the circulating blood can lead to serum sickness as manifested by glomerulonephritis, polyarteritis, urticaria, arthralgia and arthritis (Alpert, et al 1971 and Gocke, et al., 1971). Immune

complex formation involving viral agents is a common event as virus - antibody complexes probably represent natural host defense mechanism for clearing the infecting agent. However, immune complex disease may result because of either aberrant immune response against the viral agent or deficiency in clearing or degrading of these complexes. Lymphocytic choriomeningitis virus infection of the mouse has been used extensively for studying virus induced immune complex formation and disease. It has been shown in many studies (Oldstone and Dixon, 1969, 1970, 1971) that LCM virus can persist in the blood in the form of virus antibody complexes which can deposit in the kidneys of the infected mice to produce an immune complex type glomerulonephritis. Studies of mice persistently infected with lymphocytic choriomeningitis virus and lactic dehydrogenase virus (LDV) have revealed infectious immune complexes in the circulation. These complexes deposit in the renal glomeruli, arteries and choroid plexus of the brain of these mice (Oldstone and Dixon, 1969, 1971, 1972; Porter and Porter, 1971 and Oldstone, 1975). The severity of the disease is related to the strain of mouse and the relative proportions of antigen and antibody. In antigen excess the soluble complexes are formed which may be widely deposited in the kidneys, arteries and choroid plexus while in antibody excess complexes are usually cleared rapidly. Recent studies by Oldstone et al., (1980) have shown that mice

persistently infected with LCM virus mount continuous anti lymphocytic choriomeningitis antibody leading to formation and deposition of these complexes in tissues. Such mice carry infectious virus antibody complexes in the circulation. The antibody reacts specifically against the three main LCM virus structural polypeptides. The immunoglobulin eluted from the glomeruli of mice that develop glomerulonephritis was found to react specifically with LCM antigen in the complement fixation test (Oldstone and Dixon, 1969; Oldstone et al., 1975 and Buchmeier and Oldstone, 1978).

In another study (Klein et al., 1977) it was shown that the eluted immunoglobulin in the LCM infected carrier mice did not specifically react with LCM antigen as claimed by Oldstone and group.

There has not been any report in the literature to my knowledge to show that similar pathogenic immune complexes are produced by other arenaviruses. This study tries to investigate the role of immune complexes in the pathogenesis of pichinde virus infection and in the establishment and maintenance of persistent pichinde virus carrier state in mice.

1.1.9: Genetic Control of Host Susceptibility to Arenavirus Infection:

An association between the immune response and the major histocompatability (H-2) complex has been demonstra-

ted in mice infected with LCM virus (Oldstone et al., 1973). According to many investigations, H-2 is responsible for lymphocyte transformation in mixed lymphocyte cultures (Hirsch 1972), for generation of cytotoxic T-cells involved in an in vitro cell-mediated lymphocytotoxicity assay (Zinkernagel and Doherty, 1974, 1975). The H-2 products are also involved in the regulation of the immune response (Ir-loci) to a variety of antigens (Benacerraf and McDevitt, 1972), in regulation of T&B lymphoid cell cooperation (Kindred and Shreffler, 1972) and in the early phase of complement activation. Theories to explain restriction imposed by major histocompatability products on T-cell recognition of antigen have been put forward by Doherty et al., (1976) who postulated interaction of an antigen and major histocompatability products on the surface of appropriate target cells and that T-cells recognize the complex of the specific antigen and H-2 products. In the case of cellular antigens recognized by cytotoxic T-cells, the complex will consist of the specific antigen and the products of K or D region of the H-2, while for T-cell recognition of [illegible] soluble antigens, the complex would involve the products of I-region of the H-2.

Genetic influence on resistance and susceptibility of mice to infection with LCM virus has been extensively studied. It was found that relative susceptibility which is a dominant trait and relative resistance are

linked to the H-2 locus (McDevitt and Bodmer 1972 and Oldstone, 1973), In vitro and In vivo studies with LCM virus demonstrate that H-2 compatability at either the H-2 K or H-2 D locus between the target cell and the cytotoxic effector T-cell is required for maximal specific T-cell mediated lysis of LCM virus infected target cells (Doherty and Zinkernagel, 1975). While in other studies identity in either K or D locus alone is not sufficient for full lysis to occur (Forman, 1975), lack of identity between the effector cells and target cells at the K & D locus makes lysis impossible (Schmitt-Verhust and Shearer, 1975 and Marker and Anderson, 1976). Genetic influence on the immune response to LCM virus infection affect the outcome of the infection. Mice with vigorous immune response are more likely to die as a result of immune mediated pathological changes (McDevitt et al., 1974). Other studies have however indicated no association between H-2 gene complex and susceptibility to acute LCM virus disease in mice (Neustadt et al., 1978).

This work will try to find out if there is an association between the major histocompatability and susceptibility to lethal pichinde virus infection in different inbred strains of mice.

REVIEW ON PICHINDE VIRUS

1.2.0.: Historical background:

Pichinde virus belongs to the tacaribe complex of arenavirus taxonomic group. The virus was first isolated in 1965 from pooled liver, spleen and kidney tissues obtained from a rodent Oryzomys albigularis which was trapped in a forest in the Pichinde Valley of Colombia, South America (Trapido and San Martin, 1971). Some virus isolates from ectoparasites collected from viremic hosts have also been reported (Berge, 1975). A serological relationship among members of the arenavirus group was first observed in 1963 (Mettler, Casals and Shope, 1963) and similarities in morphology and morphogenesis among the arenaviruses were noted in 1969 and these similarities were the basis for grouping pichinde virus into Arenavirus taxon (Murphy, 1969 and Rowe et al., 1970).

1.2.1.: Biochemical and Biophysical Properties of Pichinde Virus:

Through the use of various drugs that inhibit nucleic acid synthesis in infected cell cultures, it has been shown that pichinde virus contains RNA genome as virus replication is not affected by DNA inhibitors such as cytosine arabinoside and 5-iododeoxyuridine (5 IUdR). The virus is enveloped with genetic information encoded in two segments of negative stranded RNA.

The larger segment (LRNA; molecular weight, 2.9×10^6) encodes a polypeptide with a molecular weight in excess of 200,000, whereas the smaller segment (S.RNA; molecular

weight 1.2×10^6) encodes the viral nucleoprotein and also a precursor of the glycoproteins which become incorporated into virions (Ramsingh et al., 1980, Vezza et al., 1980, Leung et al., 1981, Hamish et al., 1981a, 1981b). Recent findings have shown that the replication of Pichinde virus in BHK cells is tightly controlled and that the control is reflected at the levels of virus release, polypeptide synthesis, and accumulation of genomic RNAs; furthermore that this regulation is independent of host cell growth and does not require the production of defective interfering virus (Dimock, et al., 1982).

Earlier reports indicated that the negative stranded RNA of Pichinde virus contained five RNA species with sedimentation coefficients of 31S, 28S, 22S, 18S and 4-6S (Carter, Biswal and Rawls, 1973, and Carter et al, 1973). It was then suggested that the 31S and 22S RNAs were virus specific while the other RNA species were of host ribosomal origin (Carter, Biswal and Rawls, 1974; Farber and Rawls, 1975 and Leung, Ghosh and Rawls, 1977).

1.2.2.: Stability of Pichinde Virus:

Pichinde virus was found to be relatively thermostable as over 50% of the infectivity was recovered after 3 days at 4°C, 8 hours at 25°C and 2 hours at 37°C. Virus infectivity was lost at 56°C (Mifune Carter and Rawls, 1971).

Because of the presence of lipid envelope, lipid solvents such as ethyl ether inactivates the virus. The buoyant density of pichinde virus in sucrose gradient was found to be 1.18gm/cm^3 while the density in caesium chloride was 1.19-1.20gm/cm^3. The virus is stable in both sucrose and caesium chloride gradients.

1.2.3. Pichinde Virus Proteins:

Analysis of viral proteins by electrophoresis in polyacrylamide gel has revealed that pichinde virus has four structural polypeptides coded for by the virus and incorporated in the virion (Ramos, Courtney and Rawls, 1972 and Vezza et al., 1977). Two of the polypeptides are glycopeptides with molecular weights of approximately 72,000 and 34,000 daltons. The glycopeptide and polypeptide each with molecular weights of 72,000 daltons are found to be associated with the viral nucleic acid and functioning as nucleoproteins while the glycopeptide with molecular weight 34,000 daltons is an envelope constituent of the virus. The function of the 12,000 dalton polypeptide has not been determined.

1.2.4. Antigenic Properties of Pichinde Virus:

Studies have demonstrated the existence of soluble heat stable complement fixing antigen which is part of the internal component associated with the viral ribonucleic acid (Buchmeier, Gee and Rawls, 1977). In addition to the complement fixing antigens there are also

other distinct structural and non structural antigens with different localization in the virion. Complement fixing and neutralizing antibodies and those detected by fluorescent antibody technique appear to be independent (Hotchin, 1971; Trapido and San Martin, 1971). Fluorescent antibodies appear earliest (as early as a week after infection) while complement fixing antibodies appear about three weeks after infection and neutralizing antibodies not before five weeks after infection.

It is very simple to prepare potent complement fixing antisera in mice for pichinde virus with titres in the range between 1:128 and 1:512 but these sera have little neutralizing capacity when tested by the mouse neutralization test against pichinde virus (Trapido and San Martin, 1971).

Antigenic relationships among arenaviruses have been mainly detected by complement fixation test because of its simplicity and relative low hazard to personnel but recently indirect immunofluorescence is being used because it is more specific, sensitive, and less time consuming than the complement fixation test (Wulff and Lange, 1975; Wulff, Lange and Webb, 1978).

1.2.5. Pathogenesis and Immunity to Pichinde Virus:

While lymphocytic choriomeningitis virus infection of adult mice has been shown to be a classical example

of virus induced immunopathological disease, observations with pichinde virus have shown that infection of adult mice generally leads to solid immunity while infection of suckling mice results in acute lethal disease (Cole et al., 1971; Cole and Nathanson, 1973; Buchmeier and Rawls, 1977; and Murphy, Buchmeier and Rawls, 1977).

Studies with pichinde virus have indicated that intracerebral infection of suckling mice resulted in acute central nervous system disease with cerebellar ataxia, paralysis, convulsion and death. There was nothing to indicate that lesions and death were immunopathological or allergic phenomena as was the case with mice infected with Lymphocytic choriomeningitis virus. Clinical and pathological observations suggested direct cytopathogenic effect by the virus (Buchmeier and Rawls, 1977). Murphy, Buchmeier and Rawls, (1977) have shown that the spleen and liver were the major targets destroyed after acute pichinde virus infection. There was extensive and progressive necrosis in those organs which contributed to the fatal outcome. However, these authors did not find mononuclear inflammatory cell infiltrations characteristic of most necrotizing viral infections. So they suggested direct viral involvement in the pathogenicity.

1.2.6. Immune Response to Pichinde Virus Infection:

Invasion of a host by virus usually activates the immune system so that antiviral antibodies are formed and anti viral effector T-lymphocytes develop and these

immune responses in most cases lead to recovery of the host from the viral infection. Pichinde virus infection of adult mice has been found to induce high levels of humoral antibodies (Mifune et al., 1971; Trapido and San Martin, 1971; Lewis et al., 1975 and Hotchin and Kinch, 1975). However, recent studies by Buchmeier and Rawls (1977); Murphy, Buchmeier and Rawls (1977) have indicated that certain adult inbred hamster strains were susceptible to lethal pichinde virus infection.

Cell mediated immune response to pichinde virus was recently noted in certain hamster strains. Chan, Clark and Rawls (1983) using footpad swelling as an indicator of cell mediated immunity in vivo, have reported that LSH and LVG strains of hamsters developed footpad swelling when pichinde virus was given in the footpad while another hamster strain (MHA) failed to develop this foot pad swelling response because of the presence of specific suppressor cells. They have also shown that the virus replicated equally well in the footpad of both MHA and LSH hamsters and the complement fixing antibody titres to the virus produced by both strains were comparable, which indicated that the virus was a sufficient immunogen and that recognition of the virus antigens had occured. Further studies revealed that treatment of the MHA hamsters with cyclophosphamide restored the footpad swelling which proved the presence of suppressor cells in the MHA hamster strains.

Adoptive transfer studies indicated that lymphoid cells but not immune sera from foot pad inoculated MHA hamsters were capable of suppressing foot pad swelling in LSH recepients. The suppressor cells were specific for pichinde virus as suppression of swelling was not observed in recepients of MHA - sensitized cells that were injected with vesicular stomatitis virus in the foot pad.

1.2.7. Transmission of Pichinde Virus:

Certain members of the arenavirus group represent zoonotic hazards to humans as has been shown with Lymphocytic choriomeningitis virus, (Rivers and Scott, 1936; Smadel 1942; Baum et al., 1966 and Gregg, 1975). Epidemiological studies also showed the hazardous nature of Junin and Machupo viruses (Johnson et al., 1965; Kuns, 1965; Johnson, Webb and Justines, 1973). Lassa virus has also been reported endemic in parts of West Africa (Monath et al., 1974; Fabiyi and Tomori, 1975; Wulff, Fabiyi and Monath, 1975) and lassa virus produces illness in humans with high mortality rate (Buckley and Casals, 1970; Frame et al., 1970; Leifer et al., 1970 and White, 1970). Pichinde virus persistently infects Oryzomys albigularis which is the only known true carrier of pichinde virus. Pichinde virus has not been shown to cause human disease (Johnson, Webb and Justines, 1973). Although serological studies revealed evidence of pichinde virus infection among laboratory workers handling the virus, no distinct illness could be associated with

the virus infection (Buchmeier, Adam and Rawls, 1974) and the virus does not appear to be highly contagious. Studies of mice persistently infected with Pichinde virus revealed that the mechanism of transmission appeared to be the upper respiratory tract. The possibility of transmission through skin abrasions has also been suggested (Buchmeier, Adam and Rawls, 1974).

Pichinde virus is chosen for this study because it is relatively safe to handle and does not need strict containment as that of lassa virus and no recognizable illness due to pichinde virus infection has so far been reported in humans. In addition a lot has been known on its biochemical and biophysical properties and moreover highly purified infectious virus can be obtained with relative ease while similar purification procedures with LCM virus yields only small part of the original infectivity.

In addition pichinde virus study will help in future investigations on lassa fever virus infection in lower animals and man and the possibility of inducing of heterologus protection against lassa fever virus on high-risk population groups initiated by infection with pichinde virus which is not pathogenic for man.

C H A P T E R T W O

MATERIALS AND METHODS

2.1.0. Materials:

2.1.0. Viruses: Pichinde virus strain CO.N 3739 was received as freeze dried ampoules from Professor Jordi Casals of Arbo-virus Research Laboratory, Yale University, U.S.A. Lymphocytic choriomeningitis virus strain WE triple plaque purified and propagated in L-cells was received as frozen ampoules packed in ice from Dr. Fritz Lehmann-Grube, Institute of Experimental Virology and Immunology, University of Hamburg, W. Germany. Tacaribe virus strain TRII573: Sealed glass ampoules obtained from Dr. G. A. Cole, Johns Hopkins University, School of Medicine, U.S.A.

2.1.2. Cell lines: Vero cell line: a stable African green monkey kidney cell line was obtained from National Veterinary Research Institute, Vom, Nigeria.
L929 cells: A fibro-blast cell line derived from C3H mice was obtained from Medical Microbiology Department London School of Hygiene and Tropical Medicine,

London U.K. BHK-21 cells: Babyhamster kidney cell line obtained as above. Sarcoma 180 cells was also obtained as in the case of L929 cells.

2.1.3. Mice: (a) White Swiss albino mice were obtained from National Veterinary Research Institute, Vom, Nigeria.

(b) BalbC and CBA Inbred strains mice were obtained from the Nigerian Institute for Trypanosomiasis Research, Vom. AJAX and SWR/J Inbred strains mice were obtained from Medical Microbiology Dept., London School of Hygiene, London, U.K.

Guniea pigs and Rabbits were purchased from National Veterinary Research Institute, Vom. The mice were fed with commercial mouse cubes obtained from Pfizer company and were given water without restrictions. Guinea pigs and rabbits were given commercial rabbit pellets which were also obtained from Pfizer and were supplied with vegetables occasionally. Mice were bred by brother-sister mating in separate cages.

2.1.4. Sera: Antitheta serum: Lot850101 from Bionetics Laboratory products Kensington, Maryland, U.S.A.

Fetal calf serum: flow laboratories, U.K.

Complement (guinea pig Welcome reagents

Ltd., Beckenham, Kent.

Antimouse Immunoglobulins: Nordic Laboratories, U. K.

Fluorescein conjugated antimouse gamma-globulins. Nordic Laboratories.

Anti C1q: was purchased from Welcome, Co. Welcome reagents, Beckenham, England

Anti C-3 Anti IgG, IgA, IgM purchased from Behring Diagnostics, U.K.

2.1.5. Isotopes: ^{3}H thymidine 5ci/mmole) Radiochemical centre, Amersham.
^{51}Cr. sodium salt 1mci)
^{125}I. sodium iodate, 1mci)

2.1.6. Reagents: (1) Ficoll-paque solution sterile 100ml solutions

(2) α-methyl Dmannoside

(3) (Lipopolysahharide & Concanavalin A)

All were purchased from Pharmacia Fine Chemicals, Upsalla, Sweden.

Leukopack Nylon fibers) were purchased from Travenol
c2906) Laboratories
Glass wool c2401) Thetford, U.K.

Eagles medium)
Medium 199) Flow Laboratories,
Glutamine) U. K.
RPMI 1640)

Carbonyl iron powder Carboxymethyl cellulose Cyclophosphamide Fluorescein isothiocynate powder	Koch-light Laboratories Ltd., Colnbrook, England.

2.1.7. Plates & Tubes:

Microtitre plates (M29ART) Gibco-Biocult, U.K.

Microtitre tissue culture plates) Linbro disposable tissue culture Plates model Fb16-24 Tc multidish) Linbro Scientific, Hamden, Connecticut, U.S.A.

Falcon 2005 tubes) Falcon 3040 plates) Falcon 3041 lids) Beckton-Dickinson, U.K.

2.1.8. Other reagents:

Veronal complement buffer: Oxoid Ltd., U.K.

Trypsin freeze dried powder: Welcome reagent Ltd.

Tissue Tek OCT compound: Raymond Lamb, London, England.

The following media were routinely used in this study.

2.1.9. Medium for virus growth:

Sterile distilled water	80ml
5X strength Dulbecco Eagle's medium	20ml
4.4% $NaHCO_3$	5ml
Fetal calf serum (inactivated)	2ml
Tryptose phosphate broth	10ml
Penicillin and Streptomycin sol.	0.2ml

2.2.0. Medium for virus plaque assay (overlay medium)

10X strength medium 199	10ml
Sterile distilled water	40ml
4.4% Na HCO_3	5ml
Fetal calf serum (inactivated)	2ml
Penicillin and Streptomycin	0.2ml.

2.2.1. Agar overlay:

1.5% noble agar was prepared and dispensed in 50ml amounts and sterilized by autoclaving. Before use, the agar was melted and kept at 45°C water bath. Just before use equal volumes of melted agar was mixed with overlay medium.

Stock solution of neutral red was prepared 1:100 when using in overlay dilute stock solution 1:10,000 i,e 1ml stock solution to 100ml overlay.

2.2.2. Tryptose phosphate broth pH 7.3:

Tryptose phosphate broth (Bacto)	29.5gm.
demineralized water	100ml

autoclaved at 121°C for 15 minutes.

2.2.3. Stock balanced salt solution BBS):

Solution A

Anhydrous d-glucose	1grm
Calcium chloride ($Cacl_2 2H_2O$)	0.0074gm
Magnesium chloride ($Mgcl_2 6H_2O$)	0.1992gm
Potassium chloride (Kcl)	0.4026gm
Tris	17.565gm.

The above were dissolved in approximately 950ml distilled water and INHcl was added until pH is 7.6 before making the volume to one litre.

Solution B

4.14MNaCl	8.19gm. per litre.

To prepare the complete BSS, one volume of solution A was mixed with nine volumes of solution B. This solution was prepared fresh each week it was required.

2.2.4. Glycine-Hcl buffer pH 3.0:

Solution A: Glycine 2M 150.14gm/l.

Solution B: Hcl IM

222ml solution A was mixed with 100ml solution B and the pH was checked to ensure that pH 3 value was maintained.

2.2.5. 10% neutral buffered formalin (fixative):

Sodium dihydrogen phosphate $NaH_2PO_4 2H_2O$	6.50 gm.
Sodium monohydrogen phosphate $Na_2HPO_4 2H_2O$	8.15 gm.
Formaldehyde 40%	100ml
Demineralized water	900ml.

2.2.6. Phosphate buffered saline (PBS):

Sodium Chloride (Nacl)	8gm.
Potassium monohydrogen phosphate K_2HPO_4	1.21gm.
Potassium dihydrogen phosphate KH_2PO_4	0.34gm.
Demineralized water	1,000ml

The solution was autoclaved at 121°C for 15 minutes and dispensed in bottles and kept at 4°C.

2.2.7. Veronal buffer stock solution:

Sodium chloride	85gm.
5,5, diethyl barbituric acid	5.75gm.
Sodium 5,5, diethyl barbiturate	3.75gm.

Barbituric acid was dissolved in 500ml hot demineralized water and sodium barbiturate was added and the mixture was brought up to 2000ml with demineralized water. Then 1.68gm magnesium chloride ($MgCl_2H_2O$) and 0.28gm calcium chloride were added and this was autoclaved at 121°C for 15 minutes and stored at 4°C. When required the stock solution was diluted one in five with demineralized water.

2.2.8. Flavors solution:

Trisodium citrate	8gm.
D-glucose	19 gm.
Sodium chloride	4.2gm.
Demineralized water	100ml.

Autoclaved at 121°C for 10 minutes and stored at 4°C.

2.2.9. Buffers for Clq Isolation and Testing.

2.2.0. Solutions for Clq Isolation:

1. Starting solution: 0.1MEDTA in distilled water i.e. 74.448gm. . EDTA/2 litre H_2O.

2. Solution A — 0.1MEDTA pH 7.5
i.e add approx. 23ml IN NaoH to 250ml starting solution until pH 7.5.

3. Solution B — 0.005MEDTA pH 7.5
i.e 1:20 dilution of solution
:20ml of A+ 380ml distilled water.

4. Solution C — EDTA buffer pH 7.5 (approx. 50ml solution A and 70ml H_2O or add H_2O to solution A until RSC of 0.04M Nacl.

5. Solution D — EDTA buffer pH 5 i.e ± 6ml IN NaoH added to 1 litre starting solution gives EDTA pH5. For buffer pH5 add approximately 740ml H_2O to 1500ml EDTA pH5 or more exactly add water until RSC of 0.078M Nacl.

6. Solution E — Nacl 0.75M-EDTA 0.01M pH 5.
i.e I:10 dilution of solution D before adding water to the solution D and then add 2.19gm Nacl per 50ml of the solution.

7. Solution F — Nacl 0.3M-EDTA. 0.01M pH 3.5 (i.e. 1:10 dilution of solution A and then add 0.88gm Nacl/50ml.

Use sodium EDTA (Na_2H_2 EDTA molecular weight 372.24).

2.3.1. Solutions for C1q binding test:

2.3.2. Polyethylene glycol (PEG) solution (3%)

PEG M. W. 6000

PEG 3gm.)
borate buffer) PEG was dissolved in
100ml) borate buffer.

keep at 4°C refrigerator.

2.3.3. Borate buffer 0.1M pH 8.3 keep at 4°C.

Boric acid	12.366gm.
Sodium tetraborate	19.070gm
Sodium chloride	8.766gm
Distilled water	2 litres.

2.3.4. Veronal Buffered Saline VBS. keep at 4°C.

Sodium cholride	8.5gm
Magnesium chloride	0.168gm
Calcium chloride	0.028gm
Barbituric acid	0.575gm
Sodium barbitone	0.200gm.
Distilled water	1 litre pH 7.2.

2.3.5. Ethylene diamine tetra acetic acid (EDTA) buffer:

Na_2H_2 EDTA 0.2M pH 7.2

add 0.3% Tween 20.

For mouse sera 0.3M EDTA containing 0.4% Tween 20 was used.

2.3.6. Alkali Aggregate:

1. Make up 5ml of 20mg/ml solution IgG in distilled water
2. Add an equal volume of 0.2M NaoH and mix.
3. Dialyse against PBS pH7.2 at room temperature for two hours, change PBS and continue dialysis overnight.
4. Solution should be aggregated by morning (milky)
5. Store at 4°C for up to two months.

Alkaline aggregated IgG is used in the following dilutions: 2.5mg/ml, 0.625mg/ml, 0.16mg/ml, 0.04mg/ml with all dilutions being performed in NHs.

2.3.7. Michaelis Buffer pH 8.2:

Sodium diethyl barbiturate	80.28gm.
Sodium acetate	52.98gm.
EDTA (0.0005M)	9.71gm
Hcl 0.1N	750ml
Sodium azide	0.25gm

Dissolve in 4.5 litres of distilled water

Dilute 1:2 for preparing agar.

1.5gm agar in 100ml of buffer.

This buffer is used in immunoelectrophoresis to check the purity of isolated C1q using anti C1q and standard normal human serum and anti whole serum.

2.3.8. Methods Used in this Study

2.3.9. Preparation of Virus Stock:

The working stock of each virus strain was prepared by inoculating $10^3 LD_{50}$ of the virus intracerebrally to groups of 100 suckling swiss albino mice when they were about 18 hours. The litters were returned to their

mothers and observed daily for clinical symptoms.

Pool brains from moribund animals were ground in mortar and made into 20% suspension in phosphate buffered saline (PBS) containing 1% bovine albumin. The suspensions were clarified by centrifugation at 2,500rpm for 30 minutes and the supernatant containing virus was dispensed in aliquots of 0.5ml each and stored at - 70°C until used. In addition clone purified pichinde virus with infectivity titre of about $4x10^7$PFU/ml was inoculated into BHK-21 cells at multiplicity of infection of 0.001. The virus-containing supernatant fluids were harvested at intervals of 3, 4 and 5 days post infection. The fluids were individually titrated using plaque assay method as described elsewhere in this study.

2.4 0. Preparation of anti Pichinde Immune ascitic fluid:

Six Adult Swiss Albino mice were inoculated with 10^5PFU of the pichinde virus intraperitoneally (0.2ml). After seven days a second inoculum was given. After 21 days 0.2ml of the virus was inoculated and in addition 0.2ml Sarcoma 180 cells were inoculated I.P.
At day 23, another virus inoculum was given (0.2ml)
At day 25, a further virus inoculum was given (0.2ml)
Finally at day 27, 0.2ml virus was given again.
The mice were left until they developed ascites in about (4-6 weeks).. The ascitic fluids were removed and stored overnight at 4°C and then centrifuged to clarify the fluid. The fluid dispensed in 1ml amounts and stored at -20°C until tested.

2.4.1. Preparation of anti pichinde antibody in rabbits:

Antisera were prepared in rabbits by weekly intradermal injections of 1ml virus suspension emulsified in equal amounts of incomplete Freund's adjuvant over a period of three months and serum collected eight days after the last injection.

2.4.2. Extraction of complement fixing antigen from infected cell lines:

Monolayers of BHK-21 or vero cells infected with pichinde virus at multiplicity of infection of 0.001 were used. The procedure of Buchmeier et al., (1977) was followed. After 5 days post infection, the infected cells were frozen and thawed twice to lyse the cells. The cells were then sonicated for one minute three times at maximum power with cooling on ice between cycles. The sonically treated lysates were then centrifuged at 11,000 rpm for 30 minutes and the pellets discarded. The supernatant material was centrifuged at 35,000 rpm for one hour and the pellet discarded. The material remaining in the supernatant containing the soluble complement fixing antigen was used.

2.4.3. Complement fixing antigen from organs of infected mice:

The soluble complement fixing antigen from livers, Kidneys, Spleens of infected mice was extracted by sucrose-acetone extraction procedure (Gresikova and Casals, 1963 and adapted by Larsen, 1973). The organs

were aseptically removed from the mice weighed and homogenized with 4 volumes of 8.5% sucrose solution. The homogenate was added with continuous stirring to 20 volumes of cold acetone. After a few minutes agitation, it was centrifuged at 1,500g for 5 minutes and supernatant discarded.

The pellet resuspended in 20 times the volume of the acetone, stirred and incubated in ice bath for one hour. The mixture was centrifuged, the supernatant discarded and the sediment extracted a second time with acetone. After the final centrifugation, the acetone was removed by evaporation for 5 hours in ice bath. The dried powder was incubated overnight with 0.4x its volume of saline made from double distilled water. The mixture was centrifuged at 10,000rpm for one hour and the supernatant which was used as the antigen was stored at -70°C.

2.4.4. Virus titration in mice:

Stock virus titrations were conducted in mice. Serial ten fold dilutions of the virus or serum to be tested for infectivity were inoculated intracerebrally to suckling swiss albino mice using 0.03ml inoculum per mouse and at least eight mice per dilution. The LD_{50} was calculated by Reed Muench method (1938).

2.4.5. Complement fixation test:

The adapted micro complement fixation test (Casey, 1965) was used to determine C.F. titres of hyperimmune

serum obtained from rabbits or ascitic fluid of immunized mice and serum titres in mice at various intervals after infection with pichinde virus. In this method each serum was inactivated at 56°C for 30 minutes and serially diluted with veronal complement buffer in micro titer wells using spiral loops adjusted to deliver 0.025ml. An equal volume of pichinde virus antigen prepared either from suckling mouse brain or vero cells and extracted using sucrose-acetone extraction procedure (or highspeed centrifugation) and diluted 1:15 was added. A standardized dilution of guinea pig complement (1:40) was added and the plates were incubated overnight at 4°C. Sensitized sheep red blood cells (0.025ml) were added to each well and the plates thoroughly mixed and incubated at 37°C for 30 minutes and plates removed into a refrigerator for 3 hours until cells settled. The results were read visually.

2.4.6. Plaque assay:

All samples were assayed for virus content in vero cells using two plaque assay methods: a micro - plaque - forming technique where virus dilutions were mixed with approximately $5x10^5$ cell suspensions/ml and portions were distributed into wells of microtitre plates, and incubated at 37°C in CO_2 incubator for 60 minutes, then cultures were overlaid with agar in medium 199 containing inactivated fetal bovine serum plus antibiotics. The cells were incubated for 5 days at 37°C in CO_2 incubator and then a second overlay containing 0.01% neutral red was

added and incubated overnight to allow the plaques to develop.

In the second method the plaques were formed under carboxymethyl cellulose (CMC) as an overlay. Here 0.05ml of the virus dilutions or virus in sera, or virus in organs were inoculated into confluent vero cell monolayers in multi well dishes. After 60 minutes adsorption at 37°C in CO_2 incubator the cultures were overlaid with medium 199 containing final concentration of 0.7% carboxymethyl cellulose. The plates were incubated for six days and the overlay drained. The plates were stained with 0.005% crystal violet solution in saline containing 10% formalin and the plaques counted. The microtiter plates were mostly used because of convenience and economy.

2.4.7. Preparation of Antithymocyte serum:

(According to the method of Levey and Medawar 1966)

Thymus cells from CBA mice about 4 weeks old were removed and suspended in Eagles medium containing antibiotics and about $1x10^8$ cells were injected intraperitoneally into number of rabbits each rabbit weighing approximately 2.5kg. A booster dose of cells was given two weeks later and the rabbits were bled a week later, and the sera separated in readiness for inactivation, absorption and storage. The sera were inactivated at 56°C for 30 minutes and absorbed on mouse erythrocytes in a ratio of four volumes serum to one volume of packed erythrocytes. The sera were stored at -20°C for further investigation.

2.4.8. Fractionation of lymphocytes by Nylon Wool Column.

2.4.9. Preparation of nylon wool column (Julius et al, 1973):

Sterile nylon wool (obtained from Fenwal laboratories Trevanol, Thetford, England) was presoaked in a beaker containing normal saline for two hours at 37°C. Then transfered in a beaker containing double distilled water and washed at 37°C with three changes of distilled water over a period of one week. The nylon wool was spread out and dried in a 37°C incubator for two days.

About 0.5gm of the dried nylon wool was packed each into barrel of 10ml plastic syringe and the packed syringe barrels replaced in the syringe covers and autoclaved. After autoclaving the sterile nylon columns were rinsed with about 20ml Dulbecco's phosphate buffered saline (PBS) containing 5% heat inactivated fetal calf serum. The columns were drained of excess medium and then replaced in the sterile syringe covers, the lids sealed with masking tape to prevent evaporation and placed in a 37°C incubator for one hour before loading the cells.

2.4.10. Loading and separation of cells:

Spleens were dissociated with mouse tooth forceps in 60x15mm plastic petridishes. Large aggregates and connective tissue clumps were removed by settling in a test tube for 3-5 minutes. Mononuclear cells were then filtered through glass wool to remove dead cells and macrophages. A total of about $1x10^7$ - $1x10^8$ cells/2ml were loaded on to the vertical column and washed into

the nylon wool with 1ml warm dulbecco PBS containing serum. The columns were replaced in the sterile syringe covers and lids resealed and left for 40 minutes at 37°C. The columns were then washed slowly with warm (37°C) medium and the first 25ml effluent was collected into conical centrifuge tubes and the cells pelleted for 10 minutes at 4°C. Viable cells excluding trypan blue stain were counted in a hemocytometer.

2.5.4. Lymphoid cell preparation using commercial ficoll-paque:

Peripheral blood cells and spleen lymphoid cells were also purified using ficollpaque gradient centrifugation as described in the manufacturers instruction manual (Pharmacia). Heparinized blood (2ml) was mixed with equal volumes of balanced salt solution in a siliconized test tube. To each centrifuge tube 3ml of ficollpaque was added and the diluted blood was carefully layered on the ficollpaque but avoiding the mixing of blood and ficoll-paque. The tubes were centrifuged at 400xg for 40 minutes at room temperature. The upper layer was aspirated off leaving the lymphocyte layer at the interface. Using a clean sterile pasteur pipette the lymphocyte layer was transfered to a clean centrifuge tube making sure to remove most of the interface but leaving a minimum amount of ficollpaque and supernatant as removing excess ficoll-paque causes granulocyte contamination while removing excess supernatant result in unnecessary contamination by plasma

proteins. To the lymphocyte in the centrifuge tube three volumes of balanced salt solution was added and the cells were suspended gently using pasteur pipette and centrifuged at 100xg for 10 minutes at room temperature. The supernatant was removed with the aid of pasteur pipettes and the lymphocyte sediment resuspended in 6ml of balanced salt solution, and centrifuged again. The supernatant removed and cells resuspended in buffer for use.

2.5.2. Macrophage depletion:

Macrophages were depleted from the mononuclear cells obtained using Ficoll-paque or Nylon wool column by incubating the mononuclear cells preparations at a concentration of $50x10^6$ cells per dish in 100x200mm plastic petridishes for 2 hours. The dishes were occassionally rotated. The non adherent cells were aspirated by vigourously washing the plates. The phagocytic properties of the adherent cells were tested by adding various concentrations of carbonyliron prepared in Eagles MEM and incubating for 60 minutes at 37^oC in CO_2 incubator. The plates were then washed 3 times and incubated for one hour with medium alone. Macrophages were identified by their adherence property to plastic dishes and phagocytosis of carbon particles.

2.5.3. T-cell depletion:

Mononuclear cells from Ficoll-paque preparations were incubated for 30 minutes at 4^oC in MEM containing 1:50

dilution of anti θ serum. The cells were centrifuged and resuspended in MEM containing 1:10 dilution of guinea pig complement and incubated at 37°C for 30 minutes. These were centrifuged and resuspended in MEM and the number of viable cells per ml was determined using trypan blue dye. The cells were tested for invitro response to ConA and LPS.

2.5.4. B-cell depletion:

Immunoglobulin bearing cells were selectively removed from the cell suspensions by incubating on Nylon wool column (Julius et al., 1973). The efficiency of depletion was monitored by indirect immunofluorescence staining using fluorescein conjugated anti mouse immunoglobulin.

2.5.5. Cytotoxicity assay (Modification of the method of Brunner et al., 1968, Steele et al., 1973)

Semi confluent monolayers of L929 cells were infected with pichinde virus at 10^5 Pfu per 10^6 cells. After 48 hours incubation at 37°C the cells were trypsinized and labelled with chromium -51 (50 uci/10^6 cells specific activity 200-400uci/ug of chromium and incubated for 1 hour. The cells were thoroughly washed three times with serum free medium 199 and adjusted to 10^5 cells/ml. The cells were then dispensed in 0.1ml into plastic tissue culture tubes and incubated at 37°C for 3 hours to allow cells to attach. Then immune spleen cells fractionated by Nylon wool columns or Ficoll-paque method were added

at a concentration of $5x10^5$ spleen cells per 0.1ml. This gave approximately 50 spleen cells per target cell ratio which was optimum as shown in the preliminary experiments of varying spleen cell to target ratio. A similar procedure was followed for the control which had normal fractionated spleen cells added to labelled target L cells.

The tubes were incubated at 37°C. for 16 hours and then centrifuged at 1500rpm for 5 minutes. One hundred micro-litre portions were taken from each tube and placed into Luckum LP3 tubes and counted in a gamma counter. Maximum chromium-51 released was determined by freeze thawing labelled target cells three times. Spontaneous ^{51}Cr released was obtained by measuring radio activity in virus infected Cr - 51 labelled target cells to which no immune cells were added.

% cytotoxicity was calculated as follows:-

$$\% \text{ cytotoxicity} = \frac{^{51}Cr. \text{ released in the presence of immune cells.} - ^{51}Cr. \text{ released in the presence of normal cells}}{\text{Total } ^{51}Cr. \text{ incorporated from Freeze thawing} - ^{51}Cr. \text{ released in the presence of normal cells.}} \times 100$$

2.5.6. Effect of Immune spleen cells on the course of Pichinde virus Infection:

Early immune spleen cells were obtained from mice at 8-9 days post infection when maximum cytotoxicity was

demonstrated by *in vitro* cytotoxicity assay and also late immune spleen cells were taken from mice after 30 days of infection when cytotoxicity was negligible by *in vitro* methods.

The spleens (from three mice) were processed separately by Nylon wool column and the eluted T-cells were injected intraperitoneally (10^7 cells) into previously infected or infected immuno-suppressed CBA mice (12-20 mice). The controls were infected or infected immuno suppressed mice given normal spleen cells from uninfected syngeneic mice. The mice were observed for clinical symptoms and death and records were kept.

2.5.7. *Antibody mediated cytotoxicity*:

To find out if pichinde virus specific antibody can mediate cytotoxicity as reported by Oldstone *et al.* (1971) in LCM virus system, target L929 were infected with the virus and labelled with Cr-51 as described in cell mediated cytotoxicity assay. Fifty microlitres containing $5x10^5$ labelled cells were added to each glass tube, 25ulitre antiserum or plasma to be tested was added to each tube and incubated for 15 minutes. Twenty five microlitres of guinea pig complement was added and incubated for 1½ hours at room temperature after which 1ml ice cold balanced salt solution was added to stop the reaction. The tubes were centrifuged at 1200g for 15 minutes 1ml transfered to counting tubes and counted in a gamma counter and %Cr-51 released was determined.

2.5.8. Effect of Cyclophosphamide:

To determine if cyclophosphamide can moderate the mortality and covert the animals into chronic carriers as has been demonstrated with LCM virus (Gilden et al., 1972), suckling mice were divided into groups of ten each. The first group were given a single dose of cyclophosphamide (150mg/kg bodywt) three days before virus infection the second group were given multiple reduced dosage of cyclophosphamide (150, 100, 50 25mg/kg) every other day starting three days before virus infection. The third group were given only virus while the fourth group received only graded dosage of cyclophosphamide as in the second group. These mice were observed for typical symptoms of pichinde virus infection. The organs from mice dying during the period of experimentation were processed for histological examination and immunofluoresce.

2.5.9. Histology:

Tissue samples from kidneys, livers, spleen, lung, heart and brain were fixed in buffered formalin, embedded in paraffin. Sections 6um. thick were stained with haematoxylin and eosin (H&E) and periodic acid schiff reagent (PAS).

Some tissues were snap-frozen in liquid N_2 after embedding in Tissue Tek II Oct compound. These were cut in cryostat about 4um thick and stained for viral antigen using hightitre fluorescein labelled anti pichinde immunoglobulin prepared in rabbits. In addition, kidneys

were stained for possible immune complexes using fluorescein conjugated goat antimouse immunoglobulin.

2.6.0. Infectious Centre Assay:

Monolayers of vero cells were grown in 60x15mm falcon plastic petridishes using Dulbecco Eagles medium supplemented with 10% heat inactivated fetal calf serum + glutamine and antibiotics. When cell monolayers were confluent, the medium was removed and $5x10^5$ to $1x10^6$ lymphocytes were added in 0.2ml medium. After adsorption for 60 minutes at 37°C, 1ml of agar overlay in Dulbecco Eagles medium supplemented with 10% inactivated fetal calf serum plus glutamine and antibiotics was added. After the layer hardened, an additional 4ml. of 0.7% overlay was added to each plate. The plates were incubated for five days at 37°C and then overlaid with 4ml of the overlay containing 0.01% neutral red to develop the plaques. The results were expressed as number of infectious centres per 10^6 viable mononuclear cells plated.

2.6.1. Infectivity of sera from pichinde carrier mice:

The serum from each carrier CBA mouse was titrated for infectious virus by inoculating into one day old suckling swiss albino mice. Serum from each animal was diluted ten folds in PBS and 0.03ml from each dilution was inoculated intracerebrally to suckling mice using 6-8 suckling mice per dilution. The LD_{50} was calculated by Reed Muench method (1938).

2.6.2. Determination of Pichinde virus - gammaglobulin complexes in blood of pichinde carrier mice:

Pooled fresh sera from carrier mice of ages 2 months, three months and four months post infection were each treated with rabbit anti mouse gammaglobulin (3 volumes anti gammaglobulin to one volume carrier sera). The mixture was incubated at 37°C for 30 minutes and then 4°C overnight. After the overnight storage the mixture was centrifuged for 20 minutes at 2,500 rpm. The precipitation procedure was repeated until the addition of antimouse gammaglobulin causes no visible precipitate and the supernatant was free of detectable mouse gammaglobulin by immunoelectrophoresis or gel diffusion test. The amount of infectious virus remaining in the supernatant was assayed by inoculating ten-fold dilutions of the supernatant intracerebrally into suckling swiss albino mice (0.03ml 1/c) using eight mice per dilution and the LD_{50} was calculated by Reed Muench method (1938).

2.6.3. Procedure for staining tissues with H&E:

Tissues were fixed in neutral buffered formalin for at least ten days and then embedded in paraffin wax and cut about 4-6um. Sections were quickly placed in distilled water for a few seconds and stained with Haematoxylene for ten minutes and washed with distilled water for five minutes until section appeared blue by naked eye and then stained for 1-2 minutes with 1% eosin, washed quickly with distilled water and dehydrated with 95% and absolute alcohol and cleared in xylol. The sections were then mounted in glycerol mountant.

2.6.4. PAS staining:

Sections were stained for 5 minutes with periodic acid (1%), washed 2 times with potassium metabissulfite (30 second each time) containing few drops of concentrated Hcl. The sections were then stained for 5 minutes in schiff reagent and washed with potassium metabissulfite and tap water. After the above process, then the sections were stained for 3 minutes with 1% light green reagent and washed with 70%, 90% and absolute alcohol cleared with xylol and mounted on glycerol mountant (90% glycerol + 10% tris buffer).

2.6.5. Effect of Histocompatability differences on Cytotoxicity:

To determine whether histocompatability differnces between the donour effector cells and target cells affect the expressing of cytotoxicity as reported in lymphocytic choriomeningitis and vascicular stomatitis viruses (Doherty and Zinkernagel, 1975; Forman, 1975; Marker and Andersen, 1976), the cytotoxic effects of spleen cells from different strains of mice was tested against pichinde virus infected target L929 cells.(origin C3H haplotype)

The following inbred strains of mice of known H-2 haplotypes were used: AJAX ($H\text{-}2^a$), Balbc ($H\text{-}2^d$), CBA ($H\text{-}2^k$) and SWR/J($H\text{-}2^q$).

A group of at least ten mice from each strain were infected with pichinde virus by either intracerebral inoculation (.03ml $10^3 LD_{50}$) or by intraperitoneal injection (0.2ml 10^4PFU), and three mice were sacrified at intervals of 3, 6, 9, and 12 days post infection and the

spleens were processed as described earlier for cytotoxic assay using target L929 cells labelled with chromium-51. In addition the pool spleen cells from each strain was passed through Nylon wool column to separate immunoglubulin bearing B-cells and other adherent cells which adhere to the nylon wool and the T-cells which were eluted from the columns. The cytotoxic effect of each separated cell type was assessed. The effluent T-cells were treated with anti θ serum plus complement and the degree of cytotoxicity was again evaluated from each inbred strain at various intervals.

2.6.6. Virus titre and mean survival time:

The virus titres in organs and mean survival time of each mouse strain were also determined to ascertain if susceptibility to pichinde virus infection is dependent on H-2 haplotype. Here the kidneys, livers, spleens, Hearts, Lungs and brains of three mice from each strain were taken at the intervals when spleens were removed for cytotoxic assay. Each organ was homogenized into 20% suspension in PBS and centrifuged. The supernatants were each diluted ten fold and inoculated into suckling swiss mice using at least eight suckling mice per dilution. The infectivity titres were calculated by Reed Muench method(1938). The mean survival time from each inbred strain was determined by infecting twenty suckling mice from each strain with pichinde virus and observing them daily for symptoms and death for three weeks.

2.6.7. Preparation and Isolation of Clq:

Human Clq was isolated from fresh normal serum by a modification of the method of Volanakis and Stround (1972) (Zubler et al., 1976) which involved precipitation at low ionic strength in the presence of chelating agent. The Clq isolation was as follows:-

Freshly drawn blood about 150ml from single donor was allowed to clot in glass tubes for one hour at room temperature and for one hour in an ice bath. The blood was centrifuged for 30 minutes at 1,500g at 4°C. The serum obtained was centrifuged for 40 minutes at 30,000g 4°C. (Spinco 30R 17,000rpm). The free lipids from the top were removed and the remainder of the supernatant was used for isolation of Clq. The serum was immediately used.

2.6.8. Isolation of Clq:

The volume of the serum was measured and four volumes of the serum mixed with one volume of solution A (0.1M EDTA buffer pH 7.5) (see Clq buffers previously described). The mixture was incubated at 37°C for 10 minutes and then returned on ice bath. The pH was adjusted to pH 7.5 with IN NaOH. While stirring with glass rod, five volumes of solution B was added to one volume of the preceeding mixture and the mixture was incubated for one hour in the ice bath with gentle stirring for one minute in every twenty minutes,

The mixture was centrifuged at 12,000g for 30 minutes at 4°C (Spinco 30R 11,000 rpm) and the supernatant discarded, The discrete precipitate was gently resuspended in solution C twice the volume of the original serum and centrifuged. The precipitate was washed again and the final precipitate was dissolved in 12ml. of solution E (0.75M Nacl - EDTA. 01M pH5) and left overnight at 4°C. After the overnight storage, the preparation was centrifuged at 30,000g for 30 minutes at 4°C (Spinco 30R 17,000 rpm) and the supernatant was dialysed against one litre of solution D for two hours. The solution was changed and dialysis continued for a further two hours at 4°C using magnetic stirrer. After the dialysis, the preparation was centrifuged at 12,000g for 30 minutes at 4°C and precipitate was washed twice in solution D half the volume of the original serum and centrifuged at 12,000g for 30 minutes. Then the precipitate was dissolved in 3ml solution F and left on ice for two hours and then centrifuged at 30,000g for 30 minutes at 4°C (Spinco 40R 18,000 rpm). The supernatant was saved as pure Clq and dispensed in 0.5ml amounts and frozen at -70°C. One hundred microlitre amount was saved for purity check using electrophoresis.

2.6.9. Purity control:

The Clq was tested for purity by immunoelectrophoresis using 1.5% agarose in Michaelis buffer/EDTA with antiserum to whole human serum and anti Clq antiserum on a 3ml microscope slides. This was subjected to

electrophoresis in tris high resolution buffer for one hour at 100 volts and antiserum added and left in a damp chamber overnight. After the overnight storage Clq line should appear as distinct line. The yield of Clq was calculated from the optical density at 280nm.

$$\frac{O\ D}{0.682} \times \text{volume of Clq sample} = \text{mg.Clq.}$$

2.7.0. Method for Radio-Iodination of Clq:

The prepared Clq was radio iodinated by the lacto-peroxidase method (Heusser et al., 1972) to specific activity of about 2uCi of ^{125}I labelled. Clq per ug.

2.7.1. Solutions for labelling:

Solution A: Sodium iodide (NaI) 0.6mg/100ml Veronal Buffered Saline (VBS) i.e. 60mg/10ml VBS and then take 10ul to 10ml VBS.

Solution B: Lactoperoxidase 1mg/ml and stored at -70°C in aliquots of 100ul.

Solution C: Hydrogen peroxide (H_2O_2) 30% diluted 1:10,000. i.e. 10ul/10ml VBS then take 10ul add to 1ml VBS.

Solution D: Sodium iodide 6mg/ml + sodium azide 0.03mg/ml i.e. 30mgNaI/ml then 10ul to 10ml + equal volume of solution A before dilution (60mg/10ml).

Solutions were kept at 4°C before use.

2.7.2. Labelling Clq:

Labelling of Clq was done in sterilin tube put in ice bath. About 10ul ^{125}I was put into sterilin tube. Then 500ul Clq was added to it, then 10ul. Solution A, 10ul. solution B, 10ul. solution C were added consecutively and the tube was shaken carefully and left at 4°C for 15 minutes. Then 20ul. solution D was added to stop the reaction. Veronal buffered saline (VBS) was added to reach a final volume of 2ml.

2.7.3. Assessment of uptake:

Ten microlitre sample of the labelled Clq solution was counted in a gamma counter and then 0.2ml normal human serum and 2ml 20% TCA were added. The tube was shaken to mix and centrifuged at 2,500 rpm for 20 minutes. The supernatant was discarded and the radioactivity left in the precipitate was counted. The uptake of Clq fixation should be 30-50% or more. The labelled Clq solution was dialysed overnight at 4°C against one litre cold VBS and Clq dispensed in 100ul aliquots and stored at -70°C.

2.7.4. Clq binding test:

On the day of testing the labelled Clq was thawed and diluted in 5ml cold VBS containing 1% BSA (bovine serum albumin) and centrifuged at 18,000g for 40 minutes at 4°C (Spinco 40R 14,000rpm). The supernatant was used for the test.

Fifty microlitre of the test serum or controls in duplicate were mixed with 100ul 0.3M EDTA buffer and incubated at 37°C for 30 minutes. The tubes were then transfered to an ice bath and 50ul. labelled Clq solution and 1ml 3% PEG solution were added to each tube (except TCA tubes where 50ul Clq + 1ml 20% TCA were added). The mixtures were left on ice for 3 hours and then centrifuged at 2,500 rpm for 20 minutes at 4°C.

The supernatants were completely removed and discarded and the radioactivity in the precipitates was measured. The test results were compared with TCA control to determine % Clq binding. (The radioactivity in TCA precipitate was taken as 100%) Negative controls were normal serum. Positive controls were alkaline aggregated IgG diluted to various concentrations (2.5mg/ml, 0.625mg/ml, 0.16mg/ml and 0.04mg/ml.

CHAPTER THREE

3.1.0. RESULTS

3.1.1. Age related susceptibility to lethal pichinde virus infection:

The influence of mouse age on susceptibility to lethal death due to pichinde virus infection was first studied. In studies with other arenaviruses it was found that there were variations in susceptibility. The pathogeneicity of amapari, Junin and Tacaribe viruses decrease with increasing mouse age at infection (Borden et al., 1971 and Boxaca et al., 1973) while the pathogenicity of lymphocytic choriomeningitis virus (LCM) increases with increasing mouse age at infection (Hotchin, 1962; Johnson et al., 1973 and Borden et al., 1974).

In this study CBA mice of various age groups were infected with 10^3LD50 pichinde virus either by intracerebral route or intraperitoneally and these mice were observed daily for clinical signs and symptoms. The result (table 3.1) shows that pichinde virus is lethal to suckling CBA mice when inoculated by either intracerebral or intraperitoneal route. Typical symptoms of hind limb paralysis, haunched posture and shivering started to develp about five days after infection and all animals inoculated by intracerebral route were moribund by day eleven post infection while animals inoculated intraperitoneally died a little later (13-15 days post infection). Weanling and adult mice showed no apparent clinical symptoms of infection. Mortality in mice inoculated

Table 3.1: Effect of mouse age and route of inoculation on susceptibility to lethal death due to pichinde virus infection in CBA mice.

Age of mice (days)	No. inoculated and route of inoculation		No. mice dead	% Death
0-1	20	1/C	20	100
0-1	20	1/P	20	100
4	18	1/C	16	88
4	18	1/P	18	100
6	17	1/C	17	100
6	17	1/P	15	88.2
9	15	1/C	11	73.3
9	15	1/P	5	33.3
12	15	1/C	1	6.7
12	15	1/P	0	0
14	15	1/C	0	0
14	15	1/P	0	0
21	16	1/C	0	0
21	16	1/P	0	0
30	15	1/C	0	0
30	15	1/P	0	0
60	15	1/C	0	0
60	15	1/P	0	0

Legend:- 1/C = Intracerebral

1/P = Intraperitoneal.

intracerebrally between ages 0-9 days ranged from 100% to 73% while in mice two weeks or older the survival rate was 100%. Intraperitoneal infection was only effective in mice 0-4 days old while mice one week or older were less susceptible.

3.1.2. Effect of virus dose on lethality:

The next study was to determine the effect of variation of the virus dose on lethality. Doses ranging from 10^2PFU to 10^6PFU were used in inoculating separate groups of CBA mice It was found that (table 3.2) intracerebral inoculations of from 10^2 to 10^5 PFU were highly effective giving almost 100% mortality while reduced mortality was observed in dose of 10^6 PFU. The decrease in mortality in the high dose range might possibly be due to presence of defective interfering particles (DI) that could interfere with the infectivity of the normal standard virus. It was infact shown in some arenaviruses particularly lymphocytic choriomeningitis virus that DI particles mask the cell killing potential of parent virus (Welsh and Pfau, 1972; Welsh et al., 1972; Staneck et al., 1972; Pfau et al., 1973; Popescu et al., 1976 and Dutko and Pfau, 1978.

Table 3.2: Effect of Pichinde virus dose on lethality to suckling CS1 mice: Intracerebral inoculation

Experiment No.	Dose of Virus	No. dead/No. inoculated	% lethality
I	10^2 PFU	$25/_{25}$	100%
II	10^3 PFU	$30/_{30}$	100%
III	10^4 PFU	$20/_{20}$	100%
IV	10^5 PFU	$18/_{20}$	90%
V	10^6 PFU	$20/_{25}$	80%

3.1.3. The Effect of cyclophosphamide on Pichinde virus Infection:

As preliminary studies with LCM virus have shown that Cyclophosphamide was an effective immunosuppressant converting lethal acute LCM virus infection into persistent LCM carrier state, a study was under taken to find out the effect of cyclophosphamide on the course of pichinde virus infection in CBA mice. A group of mice were infected with pichinde virus by either intracerebral route or intraperitoneally and then treated with cyclophosphamide three days later. A similar group of mice were either treated with the drug alone or infected with pichinde virus and then treated with multiple but reduced dosage of the drug at three days intervals. The result (Table 3.3) indicated that infected and drug treated mice died with symptoms similar to virus infected animals but without drug treatment while the drug treatment alone did not kill the animals. This implied that the drug did not alter the susceptibility of the mice to pichinde virus infection and there was no significant difference between the virus titres in the blood and tissues of the virus infected, and virus infected drug treated mice (table 3.4). The only apparent difference between the two was in the histology of the drug treated and infected mice where there was marked depletion of lymphoid cells in the white pulp of the spleen.

This result was at variance with other studies on lymphocytic choriomeningitis virus (Rowe 1956; Rowe et al., 1963; Gilden et al., 1972 and Christeffersen et al., 1976). This result argued against immune mediated nature of pichinde virus infection. However, it was possible that cyclophosphamide affected only one component of the immune response that is the antibody mediated functions without affecting the cellular aspects which are important in the immunopathology of pichinde virus infection.

Table 3.3: Effect of Cyclophosphamide on the course of pichinde virus infection:

Drug dose and route	Virus route and age of mice	No. mice inoculated	No. dead	% death
100mg I/P	I/C 1 day old	20	20	100
100mg I/P	I/P 1 day old	20	20	100
100mg I/P	I/C 4 days old	15	15	100
100mg I/P	I/P 4 days old	15	13	86.6
None	I/C 1 day	20	20	100
None	I/P 1 day	18	17	94.4
None	I/C 4 days	15	15	100
None	I/P 4 days	14	14	100
100mg I/P	none 1 day	15	0	0
100,50	none 1 day	15	1	6.8
100,50,50	I/C 4 days	16	8	50

Legend: I/P = Intraperitoneal
I/C = Intracerebral
100,50,50mg = Cy. dose of 100mg and then 50mg doses repeated at three days interval.

Table 3.4: Infectivity titre of cyclophosphamide treated and control infected mice with Pichinde virus. (1-6 days old CBA mice inoculated i/c)

Days post infection	Cyclophosphamide treated and infected				Infected mice			
	Brain	Liver	Spleen	Blood	Brain	Liver	Spleen	Blood
3	$10^{2.1}$	$10^{2.6}$	$<10^{1}$	$10^{4.7}$	$10^{1.3}$	$10^{2.3}$	$<10^{1}$	$10^{4.5}$
4	$10^{4.0}$	$10^{4.3}$	$10^{1.9}$	$10^{5.5}$	$10^{3.5}$	$10^{4.1}$	$10^{1.2}$	$10^{5.1}$
5	$10^{4.5}$	$10^{5.1}$	$10^{2.3}$	$10^{5.1}$	$10^{4.2}$	$10^{5.5}$	$10^{1.9}$	$10^{4.6}$
6	$10^{4.3}$	$10^{4.7}$	$10^{1.3}$	-	$10^{4.2}$	$10^{5.1}$	$10^{1.6}$	-
7	-	-	-	$10^{2.1}$	-	-	-	$10^{1.6}$
8	$10^{3.7}$	$10^{4.0}$	$<10^{1}$	$<10^{1}$	$10^{3.5}$	$10^{4.2}$	$10^{1.1}$	-
9	-	$10^{3.2}$	-	-	-	$10^{2.9}$	$<10^{1}$	-
10	-	-	-	-	-	-	-	-

(-) = Not tested

3.1.4. Role of cell mediated Immune response in the pathogenesis of acute Pichinde virus infection in CBA mice.

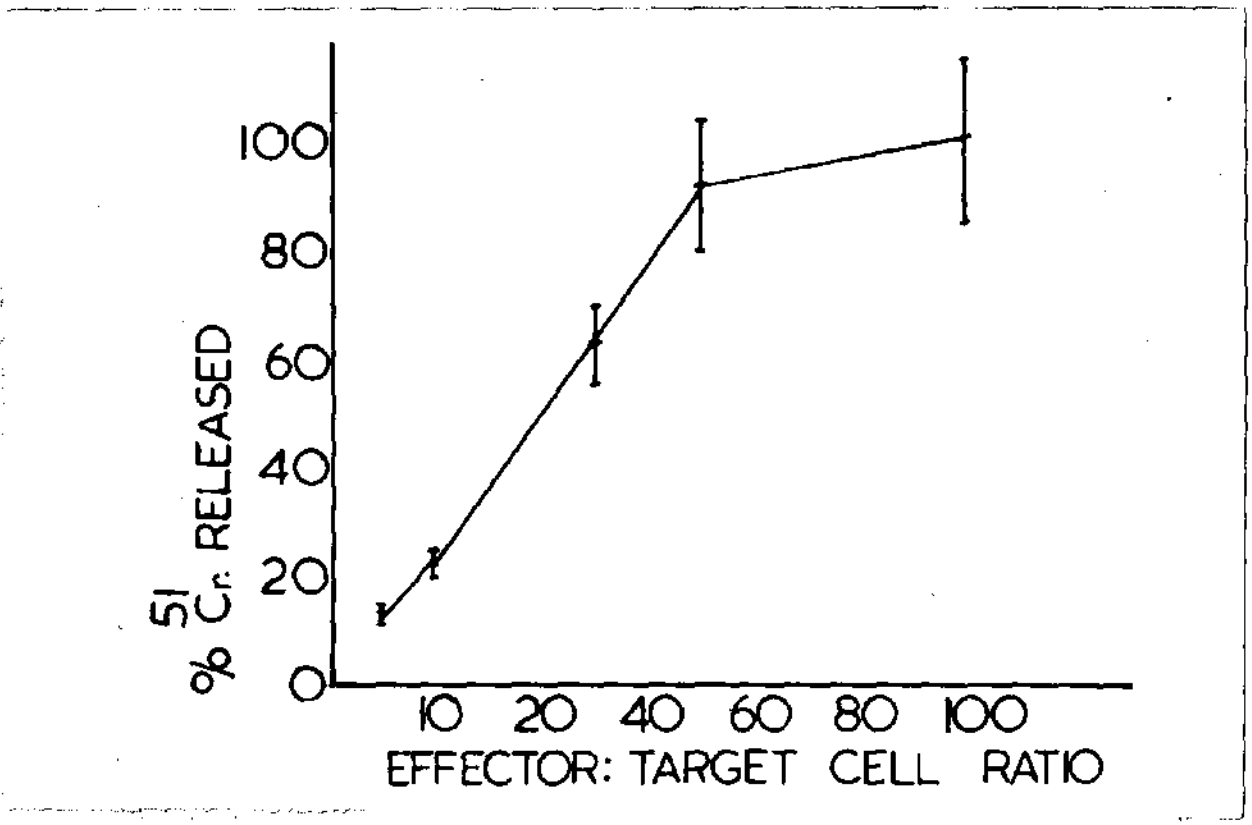
5.1.5. Introduction.

In lymphocytic choriomeningitis infection of mice, immune T-cells have been shown to mediate LCM virus induced immunopathology in vivo (Cole et al., 1972; Doherty and Zinkernagel, 1974 and Johnson and Cole, 1975). In other studies (Mims and Blanden, 1972; Johnson and Cole, 1975), it has been shown that primary effector T-cells could protect normal mice from developing lethal meningitis when these effector cells were given 18-24 hrs. prior to intracerebral challenge with LCM virus. Reduction of virus titres was demonstrated in spleen, liver and lungs of animals when T-cells from spleens of immunized mice were transplanted into infected recipients.

The importance of cellular immunity in the recovery from certain viral infections has been well-established particularly in T-cell deficient individuals (W.H.O. Tech. Report, 1973). In order to determine the role cell mediated immune response plays in the pathogenesis of mice acutely or chronically infected with pichinde virus, the following preliminary experiments were carried out:- Groups of mice 1-6 days old and 9-14 days old were each given $10^4 LD_{50}$ pichinde virus either intracerabrally or intraperitoneally and at intervals of 3, 6, 9, 12, 15, 30 and 60 days post infection spleen cells from each

group (pool spleen from 2-3 animals) were processed at the same time by nylon wool column method and the effluent T-cells were checked for specific cytotoxicity using chromium labelled target L929 cells and varying the effector to target cell ratio. The result (Fig. 3.1) showed progressive increase in target cell lysis reaching optimum when the effector to target ratio was 50:1. Therefore this ratio was taken in all subsequent experiments in this study.

The maximum specific T-cell mediated lysis was obtained 9 days after acute pichinde virus infection (fig. 3.2) and dropped significantly after this period. It was during this period of maximum cytotoxicity that clinical symptoms were apparent in the acutely infected animals and when histopathological changes in vital organs such as brain, liver and kidneys were prominent.


100
80
60
40
20
0
% ^{51}Cr RELEASED
10 20 40 60 80 100
EFFECTOR: TARGET CELL RATIO

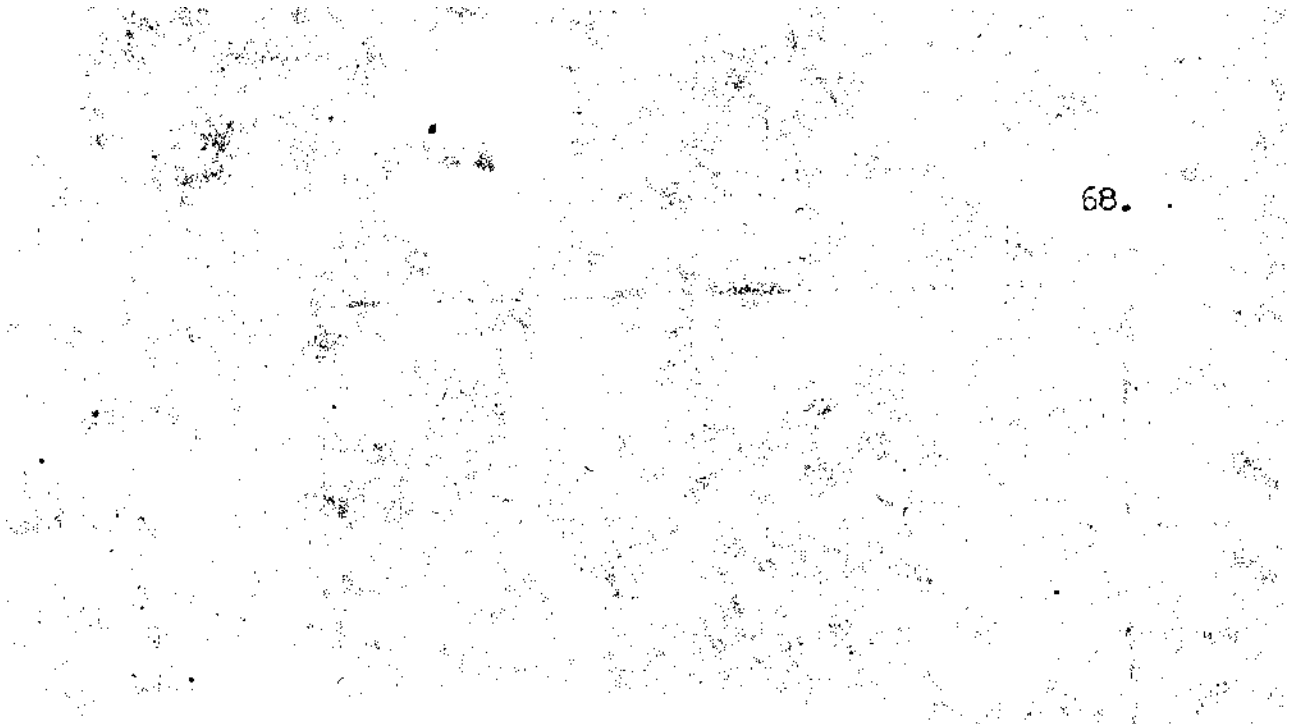


Fig. 3.1: Effect of Immune spleen to target cell ratio on ^{51}Cr released from pichinde virus infected target L929 cell.(1-6 days old mice inoculated intracerebrally).
Chromium released by normal spleen cells at the same ratio has been sub-tracted. Each point represents the mean of 4 replicates and vertical bars the $\pm$ SEM.

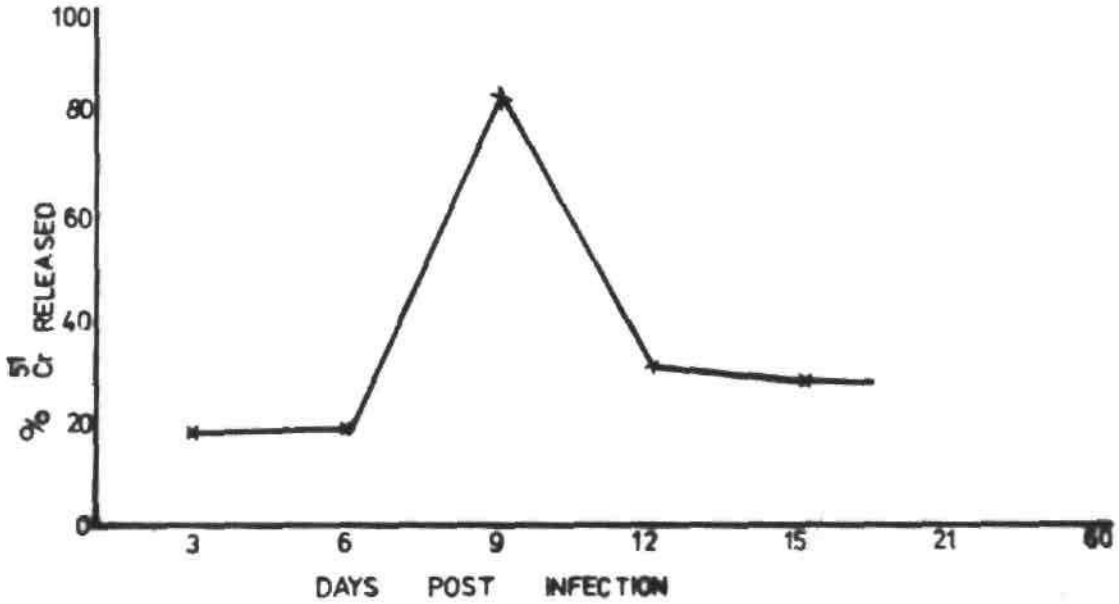

100
80
60
40
20
0
% 51Cr RELEASED
3
6
9
12
15
21
60
DAYS POST INFECTION

Fig. 3.2: Sequential development of cytotoxic activity in spleen of CBA mice acutely infected with 1000 LD_{50} Pichinde virus.
(1-6 days old mice inoculated intracerebrally)

3.1.6. Virus concentration organs of acutely infected mice:

The relationship between mortality and cell mediated response with the extent of virus infection of organs of these mice were investigated by removing from three mice of each age group, the brains, livers, kidneys, hearts, lungs and spleens at different time intervals after infection. Portions of each of these organs were processed for histology while the remainders were each titrated for virus content. The results obtained in this experiment (fig. 3.3)strongly demonstrated that mice infected during the first week of life developed high virus titres in the brains, livers and other organs. Titres being maximum when the CMI was approaching its peak as shown by in vitro cytotoxicity assay (table 3.6). In contrast mice infected when older than nine days failed to show the high virus contents in those organs (table 3.5) and there was only transient viremia in these animals. (Table 3.5). The CMI in those mice was also highest at day 9 post infection (Table 3.7).

Table 3.5 Infectivity titre of CBA mice acutely infected with Pichinde virus. (9-14 days old mice inoculated i/c)

Days post infection	Brain	Liver	Spleen	Kidney	Blood
3	$10^{2.5}$	$10^{1.1}$	$<10^{1}$	$10^{1.1}$	$10^{4.3}$
6	$10^{1.2}$	$10^{3.0}$	$10^{2.5}$	$10^{3.8}$	$10^{4.7}$
9	$10^{4.7}$	$10^{5.1}$	$10^{4.8}$	$10^{3.5}$	$10^{4.1}$
12	$10^{2.4}$	$10^{1.7}$	$10^{1.2}$	$10^{1.6}$	$10^{1.1}$
15	$10^{1.7}$	$10^{1.2}$	$<10^{1}$	$<10^{1}$	$<10^{1}$
30	$<10^{1}$	$<10^{1}$	$<10^{1}$	$<10^{1}$	$<10^{1}$
60	$<10^{1}$	$<10^{1}$	$<10^{1}$	$<10^{1}$	$<10^{1}$

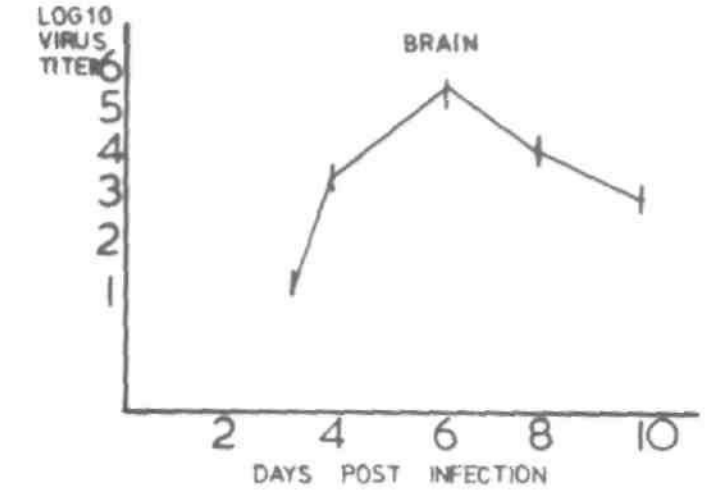
Table 3.6 Cytotoxicity assay in Suckling mice acutely infected with pichinde virus. (1-6 days old CBA mice infected i/c).

(Each experiment represents mean cytotoxicity of four replicates).

Exp. No.	Days spleen taken post infection	% cytotoxicity
1	6	18.33
2	6	19.69
3	6	15.02
4	9	61.02
5	9	59.98
6	9	48.00
7	9	89.93
8	12	34.86
9	12	39.25
10	15	20.41
11	15	16.95

Table 3.7 : Cytotoxicity assay in pichinde infected Immune mice. (9-14 days old CBA mice infected i/c).

Exp. No.	Days spleen taken post infection	% ^{51}cr. released
1	3	26.82
2	3	23.48
3	6	26.12
4	6	25.70
5	9	70.61
6	9	86.75
7	9	92.37
8	9	85.51
9	12	24.96
10	12	25.75
11	12	26.33
12	15	21.00
13	15	19.40
14	30	10.90
15	30	11.60
16	60	11.00
17	60	9.40


LOG10
VIRUS
TITER
6
5
4
3
2
1
BRAIN
2
4
6
8
10
DAYS POST INFECTION

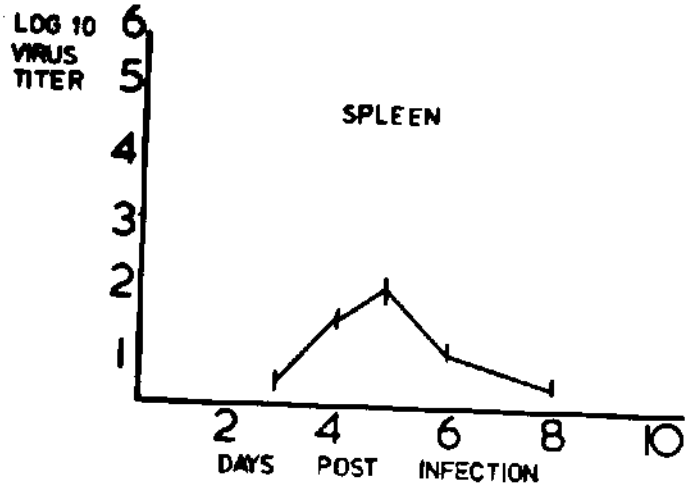

LOG 10
VIRUS
TITER
6
5
4
3
2
1
LIVER
2
4
6
8
10
DAYS POST INFECTION

Fig. 3.3 a & b: Log virus titre in the brain and liver of mice acutely infected with pichinde virus:

(a) Brain

(b) Liver

(1-6 days old CBA mice inoculated i/c)


LOG 10
VIRUS
TITER
6
5
4
3
2
1
SPLEEN
2
4
6
8
10
DAYS POST INFECTION

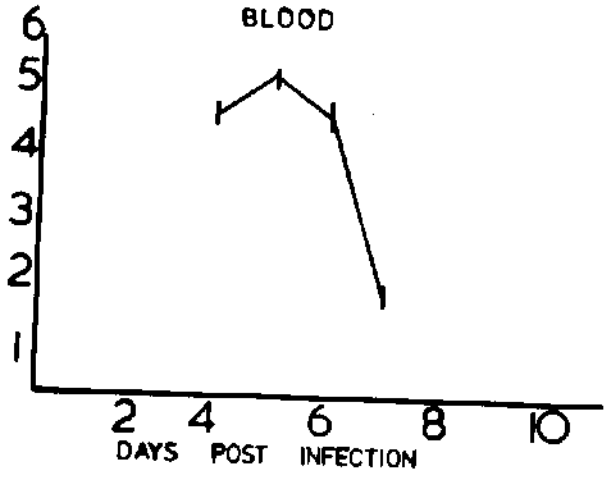
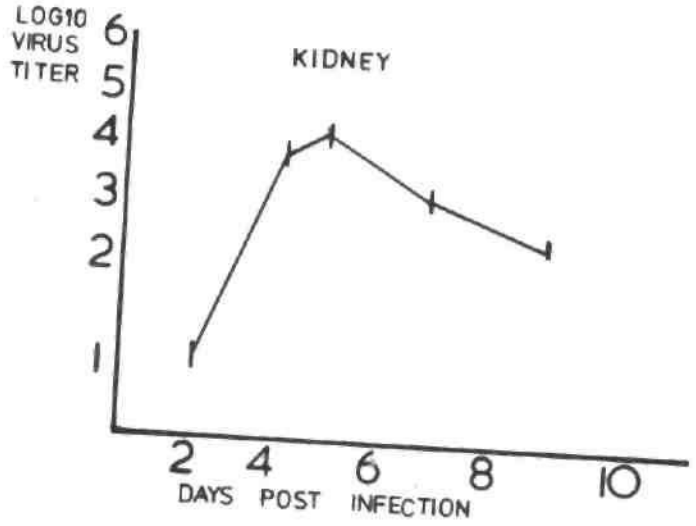

BLOOD
6
5
4
3
2
1
2
4
6
8
10
DAYS POST INFECTION

Fig. 3.3 c & d: Log virus titre in the spleen and blood of mice acutely infected with pichinde virus:

(c) Spleen

(d) Blood.

(1-6 days old CBA mice infected i/p)


LOG10
VIRUS
TITER
6
5
4
3
2
1
KIDNEY
2
4
6
8
10
DAYS POST INFECTION

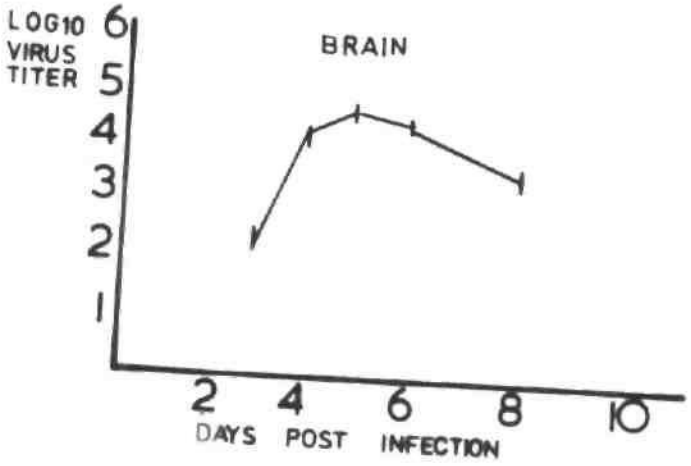

LOG10
VIRUS
TITER
6
5
4
3
2
1
BRAIN
2
4
6
8
10
DAYS POST INFECTION

Fig. 3.4 a & b: Log virus titre in the kidneys and brain of mice acutely infected with pichinde virus:

(e) Kidney

(f) Brain

(1-6 days CBA mice infected i/p)

3.1.7. Organ virus titration and staining:

Peak virus titres were maximum between 5-6 days post infection (fig. 3.3a, b, c, d) and declined thereafter. Blood virus titre (viremia) was maximum at about 5 days post infection (fig. 3.3d). The highest virus titres were attained in the brain with $10^{5.5}$ PFU/gm at day 6 post infection (fig. 3.3a) infectious virus persisted only in carrier mice.

Immunofluorescent staining for viral antigen showed progressive increase in the intensity of viral antigen as the infection progressed.

3.1.8. Histopathology of acutely infected CBA mice:

Histopathological examination of the tissues demonstrated that inflammatory reactions were maximal when clinical signs were apparent. The lesions varied as the infection progressed.

Liver:

There was widespread lymphocytic cellular infiltration of the parenchyma and periportal areas (plate 3.1). The Kupffer cells showed moderate degree of swelling and the sinusoids had clusters of leucocytes. Other changes included large, irregularly shaped hepatocytes (giant cells) with bizarre nuclei.

Spleen:

The white pulp showed areas of necrosis with accumulation of eosinophilic amorphous debris. There was evidence of extra medullary haematopoiesis and large numbers of megakaryocytes (plate 3.2).

Kidney:

The changes included marked coagulation necrosis involving more the distal than the proximal convoluted tubules (plate 3.3). Some of these tubules contained amorphorus eosinophilic casts or debris in the lumina. Some of the tubules without necrosis had swollen epithelium bulging into the lumina.

Brain:

The organ was congested. The choroid epithelium was oedematous and markedly infiltrated with mononuclear cells and some blood vessels were cuffed with mononuclear cells. There were marked astrocytic and microglial reactions (plate 3.4). The leptomeninges showed mild infiltration with mononuclear cells mainly lymphocytes.

Lung:

Alveolar septal thickening resulting in narrowing of the lumina was present (plate 3.5). Some vasculature had mild hyalinization.

Heart:

There was no significant change from normal.

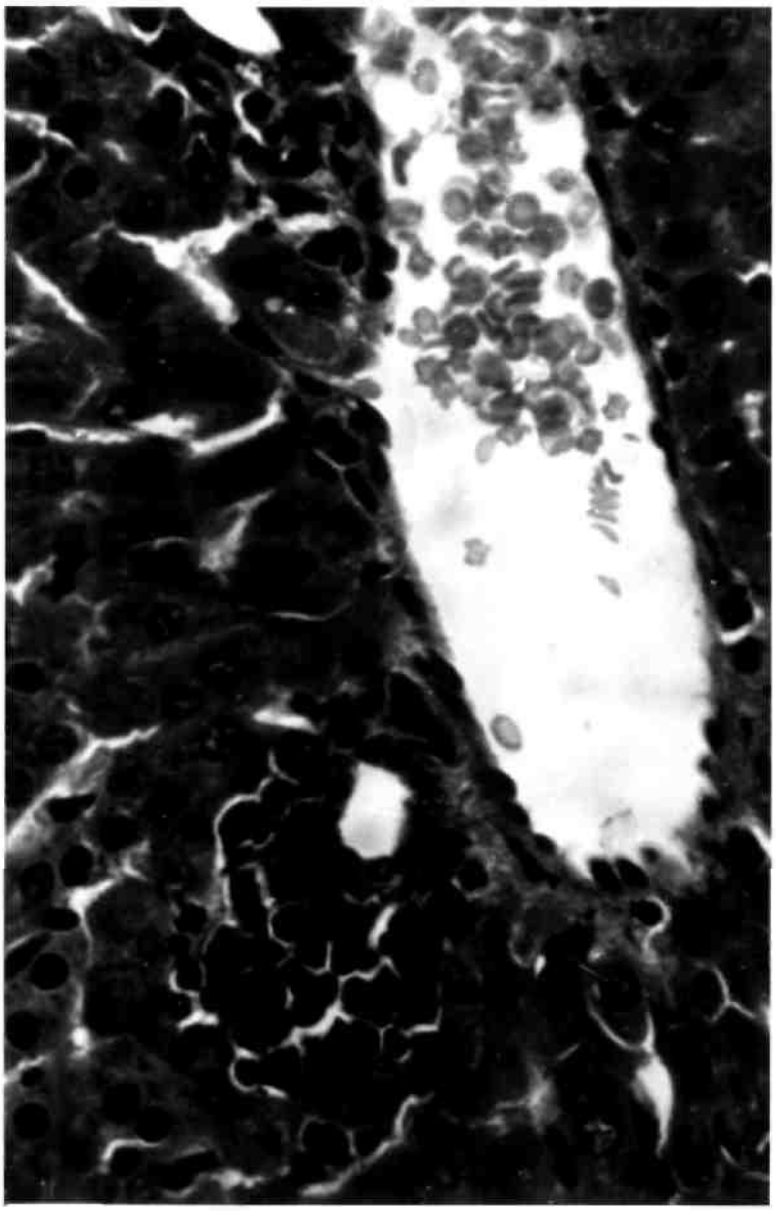


Plate 3.1 a: Photomicrograph of liver of mouse infected with Pichinde virus.

Note the lymphocytic infiltration around the bile duct. H & E stain x 400.

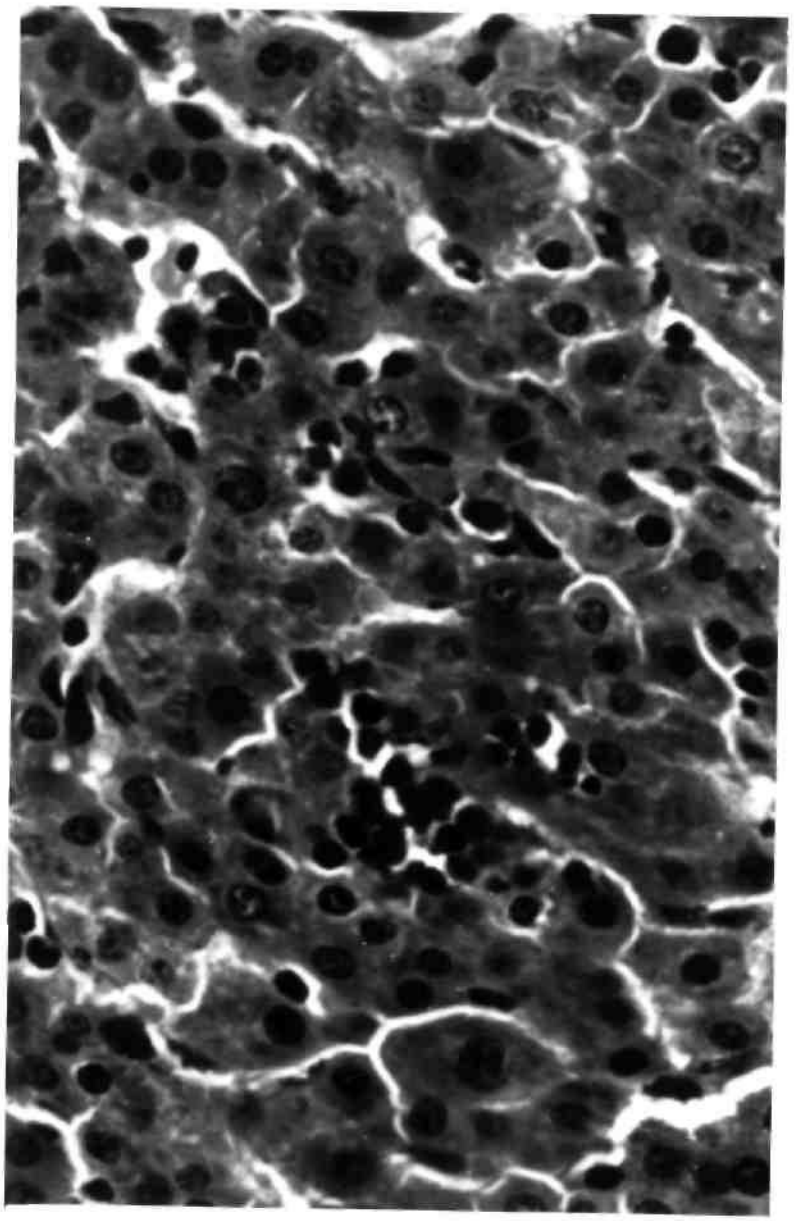


Plate 3.1 b: Photomicrograph of liver of mouse infected with Tichinde virus showing mononuclear cellular infiltration.

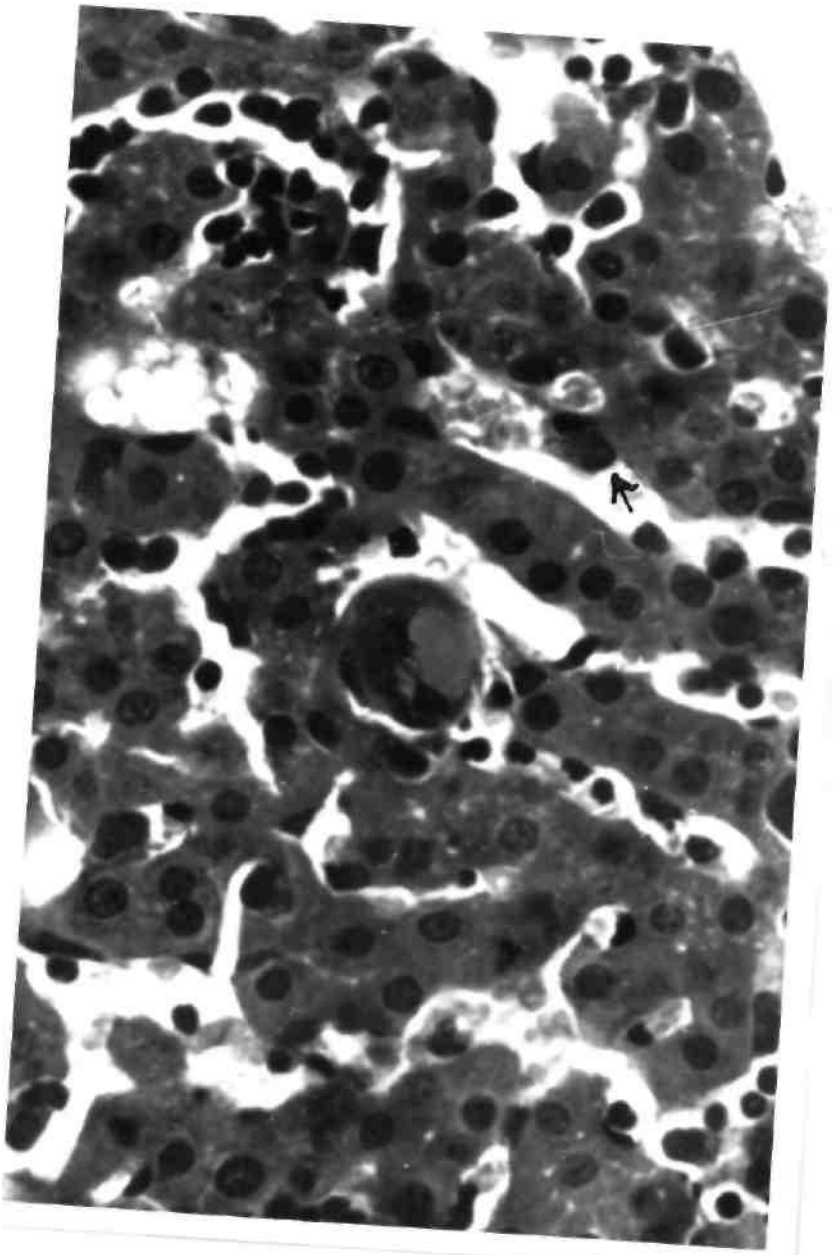


Plate 3.1c: Photomicrograph of liver of mouse infected with Pichinde virus. Note: The swollen hepatocyte (giant cell) and kupffer cells (black arrow). H & E stain x 400.

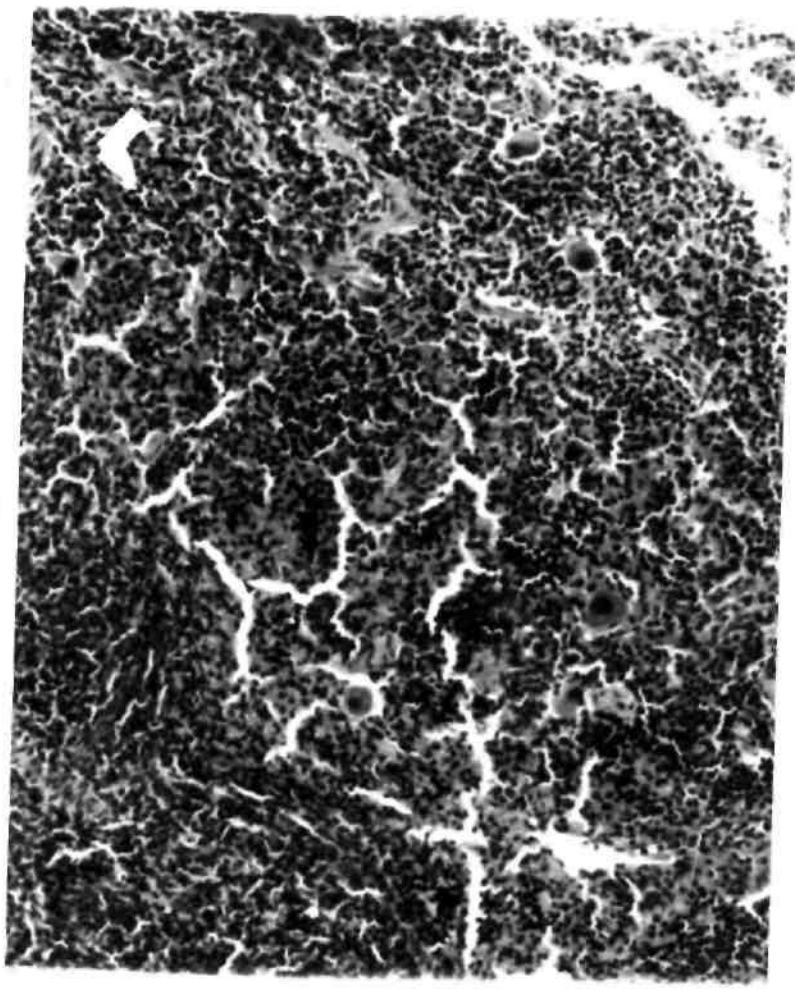


Plate 3.2: Photomicrograph of spleen of mouse infected with Pichinde virus. Note necrosis of the white pulp and giant cells.
H & E stain x 100.

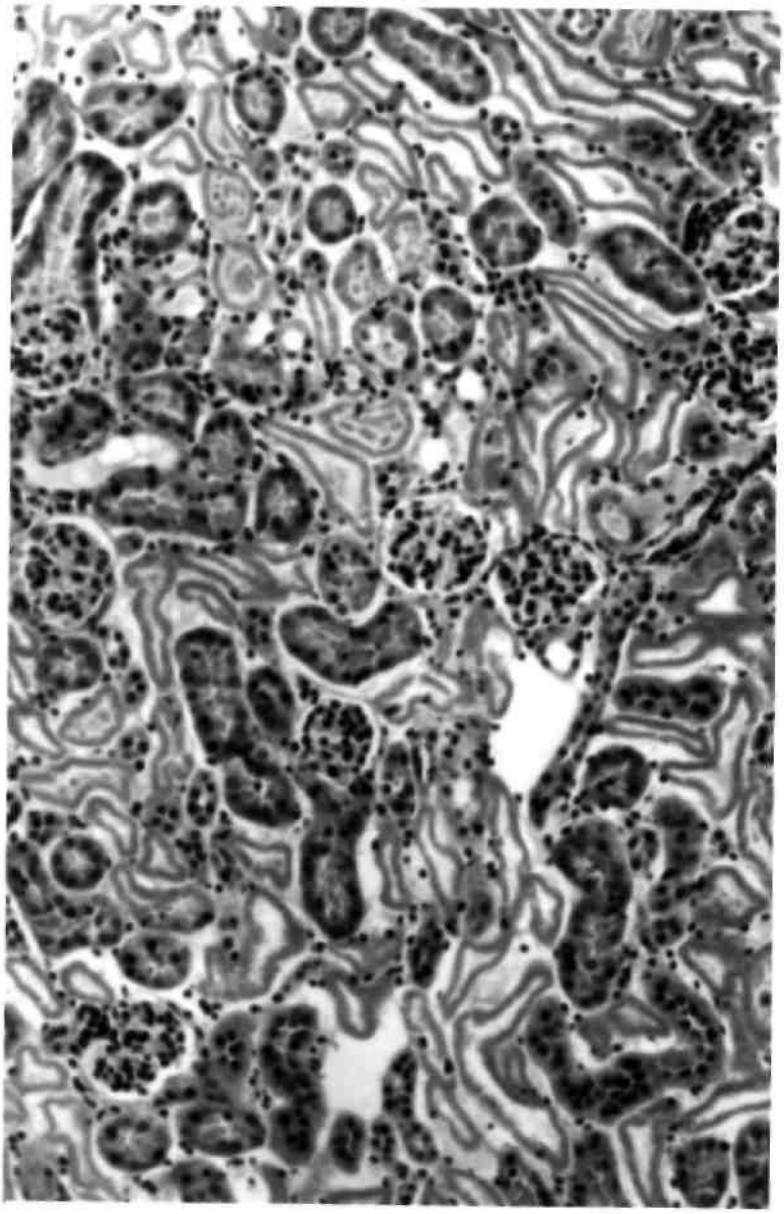


Plate 3.3a: Photomicrograph of kidney of mouse infected with Tichinde virus. Note coagulation necrosis of the convoluted tubules.

H & E Stain x 100

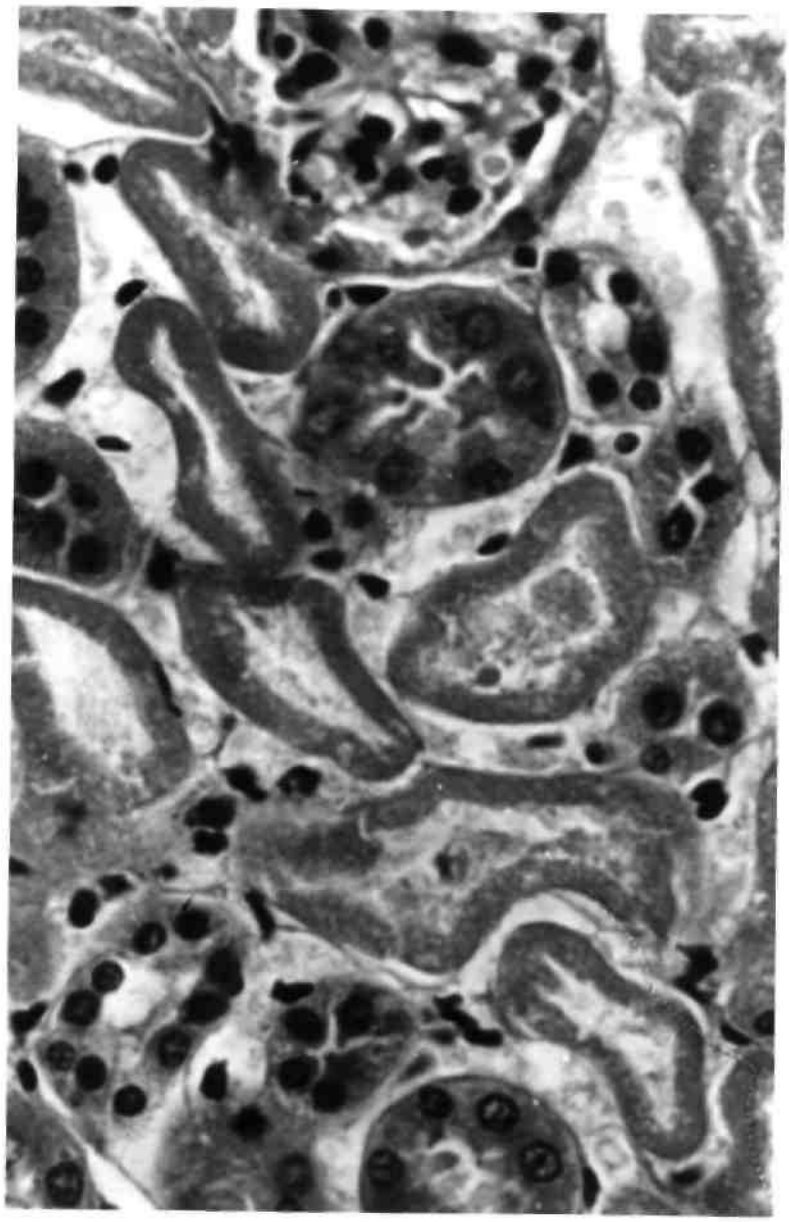




Plate 3.3b: Photomicrograph of kidney of mouse infected with Kichinda virus. Note coagulation necrosis of the convoluted tubules.

H & E Stain x 400

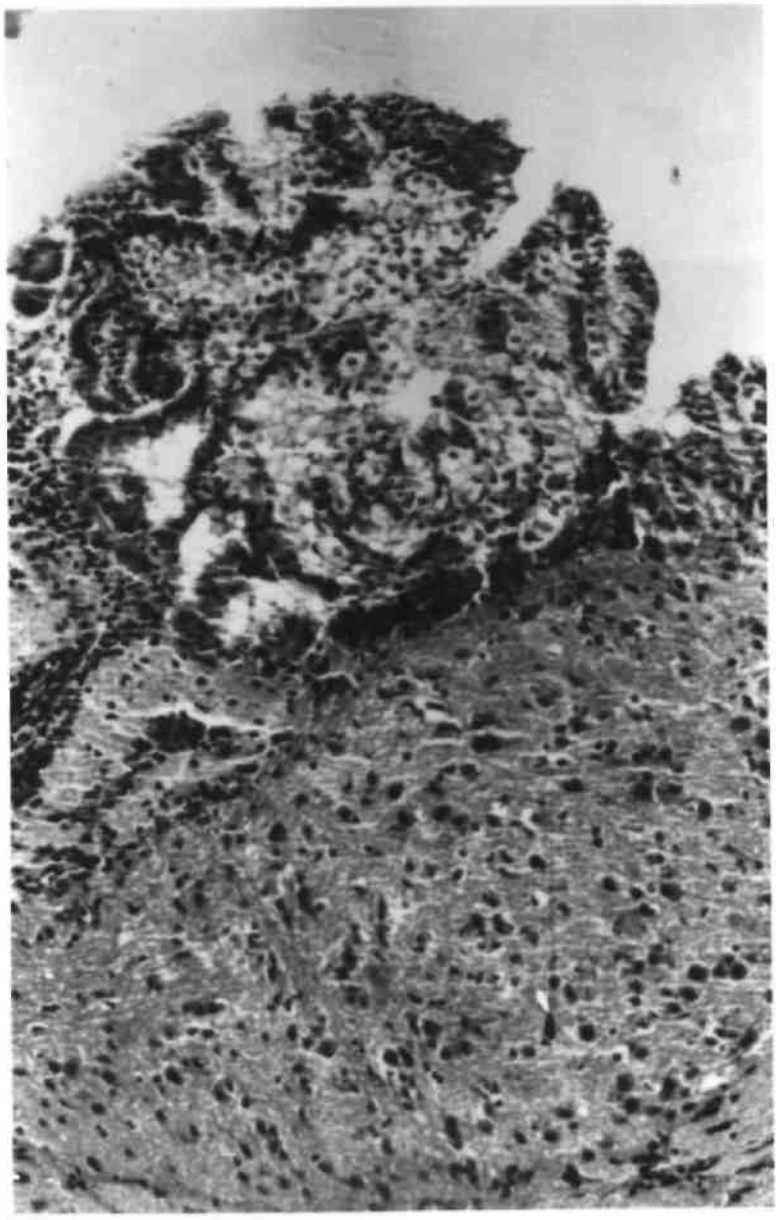




Plate 3.4a: Photomicrograph of brain of mouse infected with Tichinde virus The choroidal epithelium was edematous and infiltrated with mononuclear cells.

H & E stain x 400.



Plate 3.4b: Photomicrograph of brain of mouse infected with Pichinde virus. Note perivascular cuffing with mononuclear cells (top left corner) and choroid epithelium infiltrated with some mononuclear cells. (top)

H & E stain x 400.

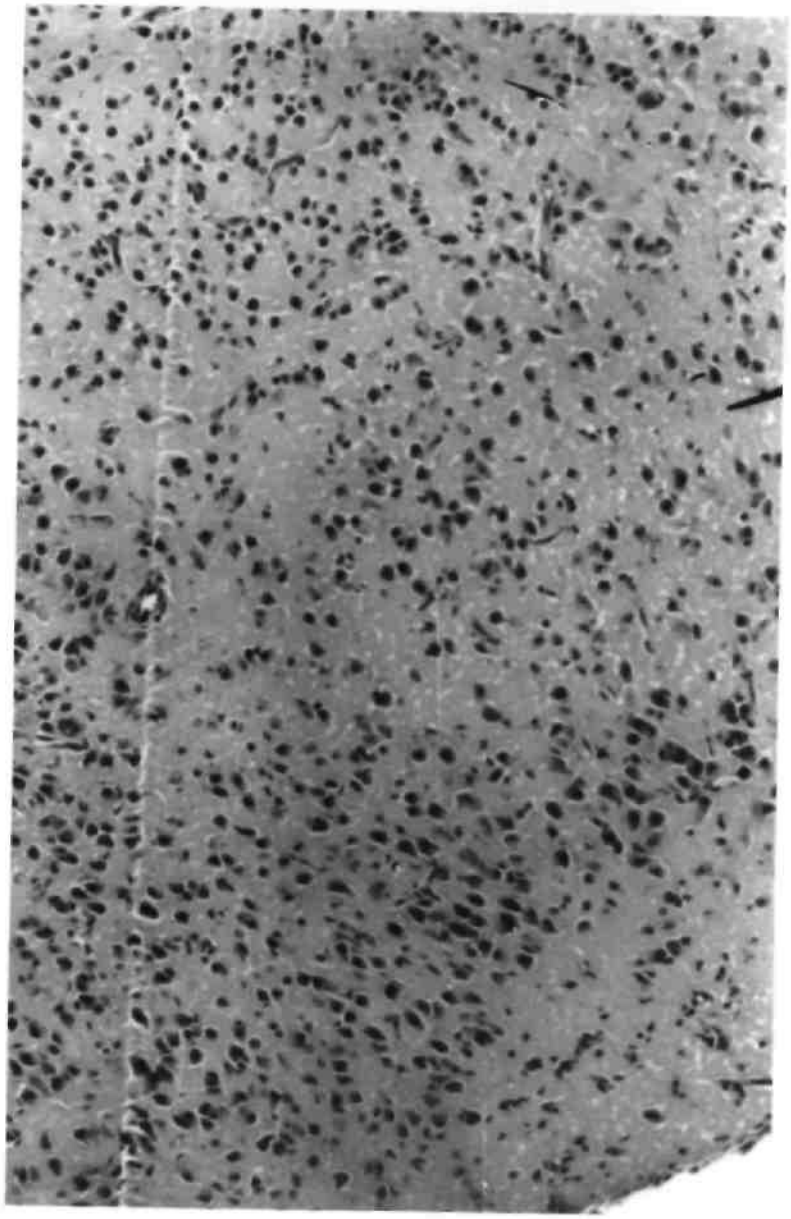


Plate 3.4c: Photomicrograph of brain of mouse infected with Pichinde virus. Showing marked astrocytic and microglial reaction.

H & E stain x 100.

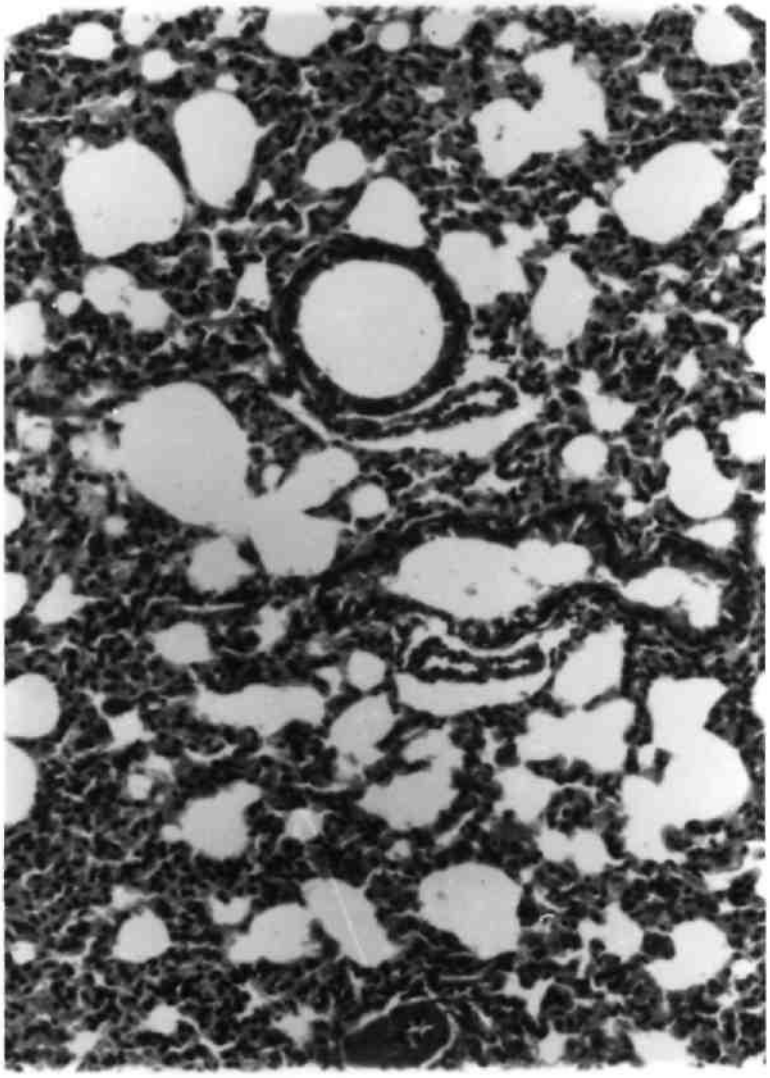


Plate 3.5: Photomicrograph of lung of mouse infected with Pichinde virus. Note septal thickening resulting in narrowing of the alveolar lumens.

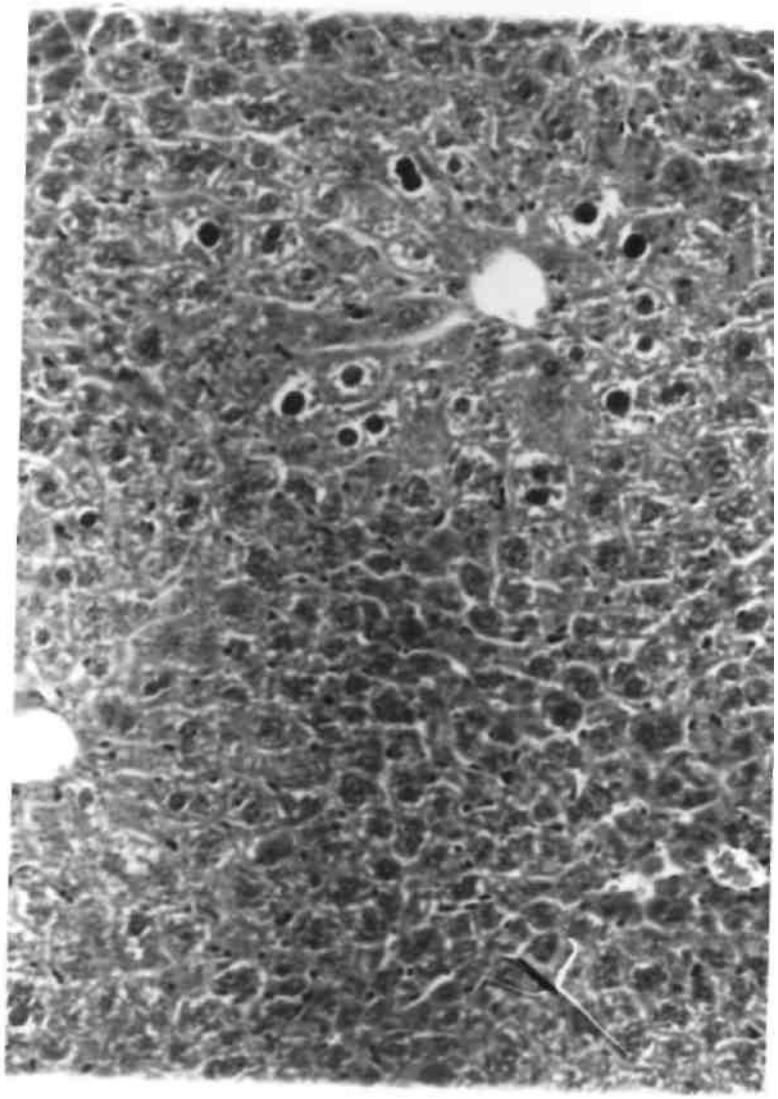




Plate 3.6a: Photomicrograph of liver of mouse infected with Pichinde virus. Note some pyknotic nuclei around the central vein.
H & E stain x 100.

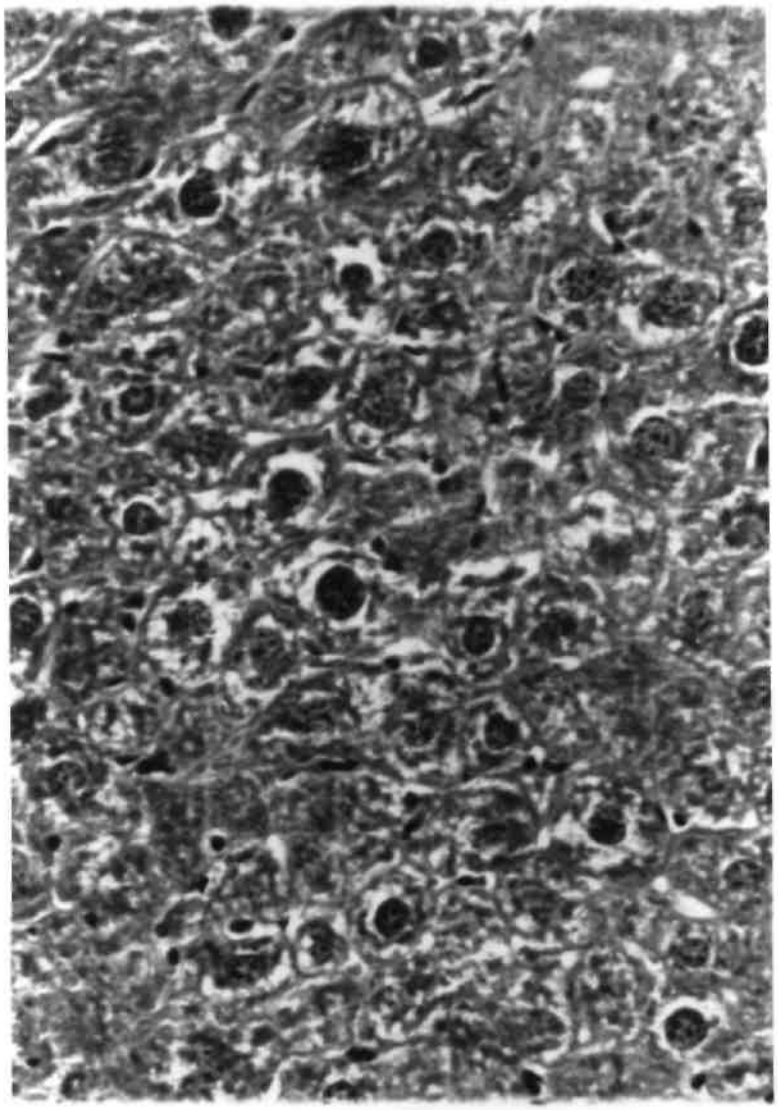


Plate 3.6b: Photomicrograph of liver of mouse infected with Pichinde virus. Note hepatocytes with swollen nuclei and which had undergone hydropic degeneration.

H & E stain x 400.

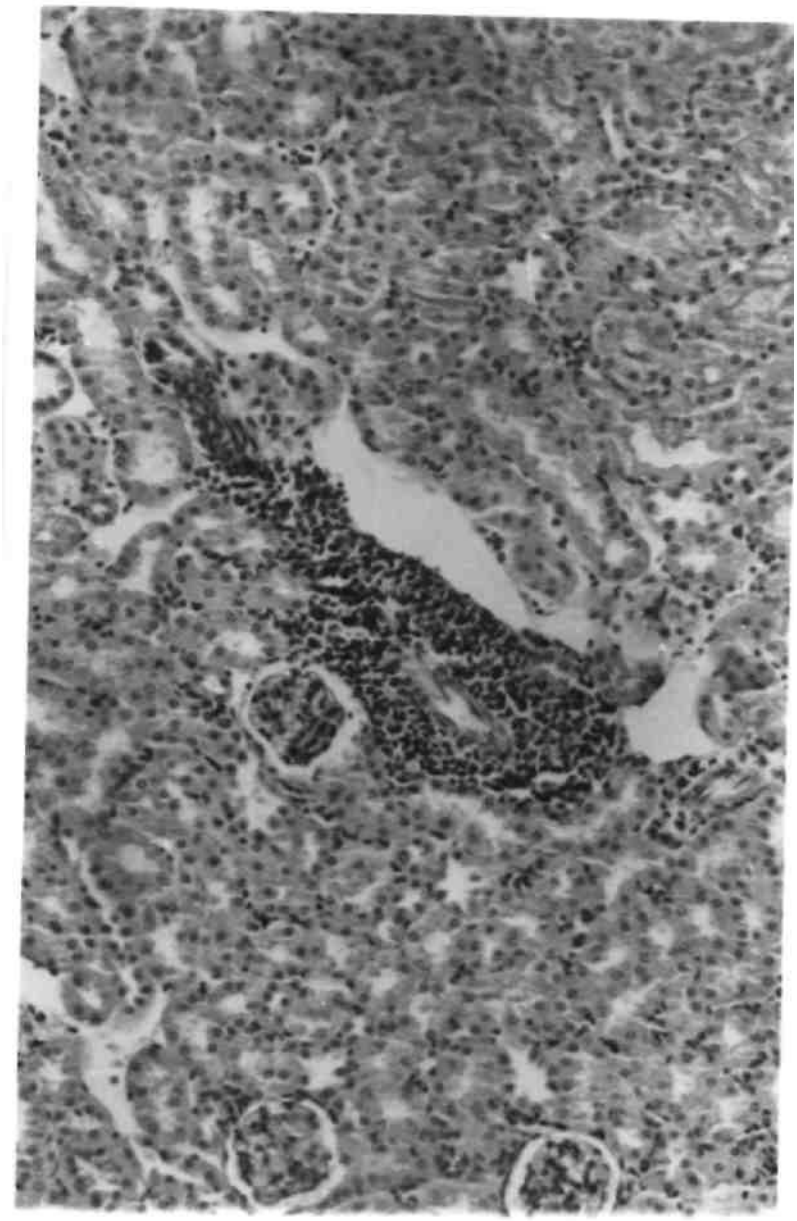


Plate 3.7 a: Photomicrograph of kidney of mouse infected with Kichinde virus showing perivascular cuffing with lymphocytic cells.

H & E stain x 100.

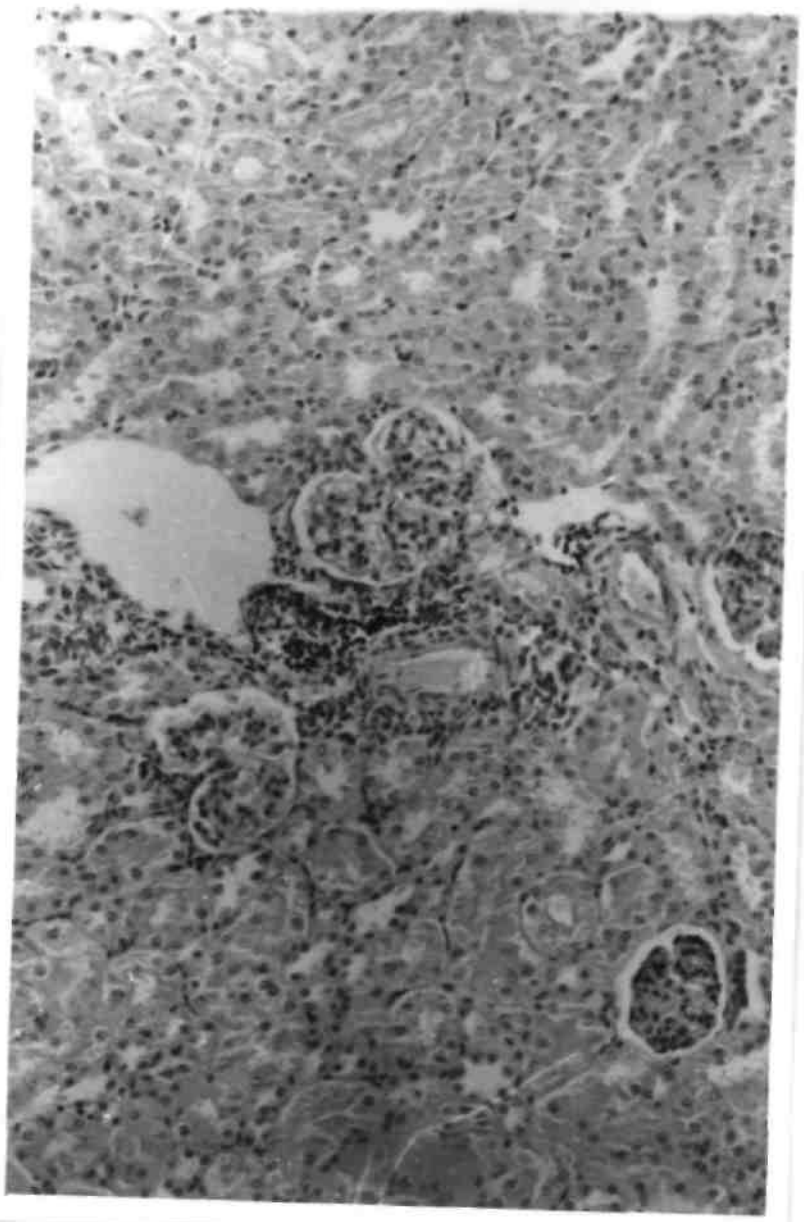
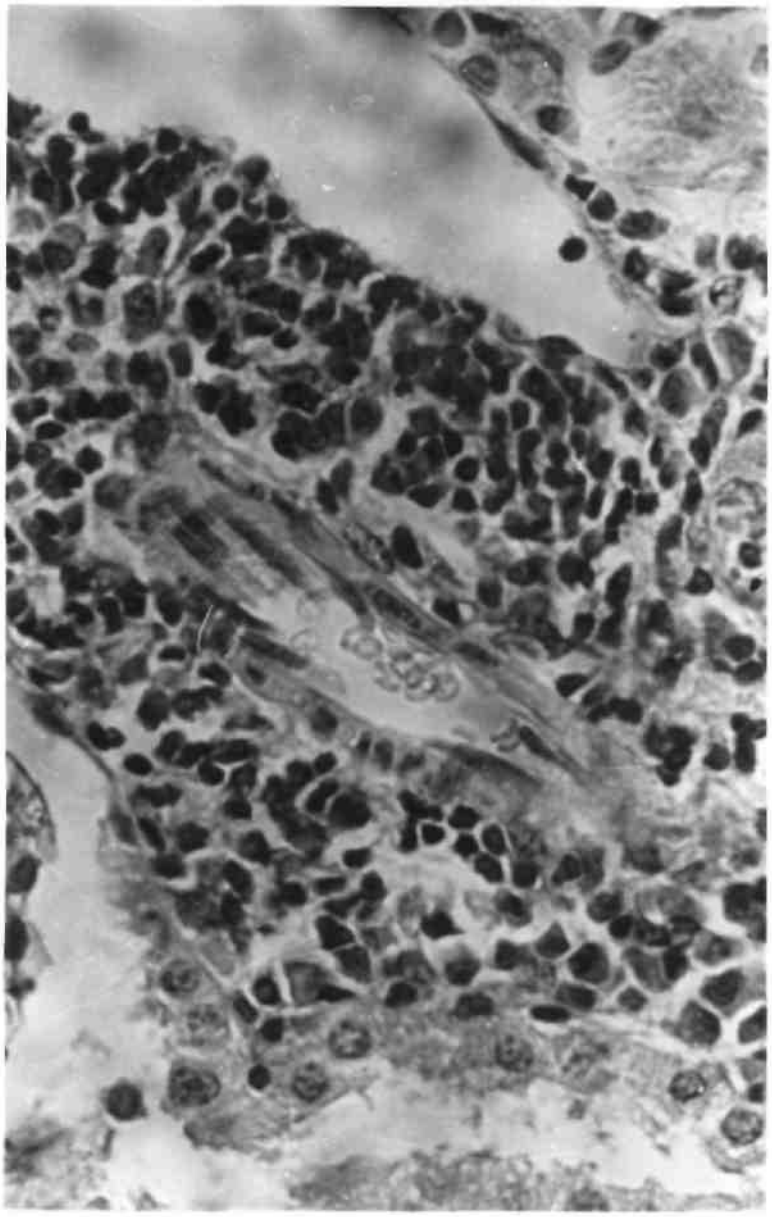


Plate 3.7 D: Photomicrograph of kidney of mouse infected with Kichinde virus showing perivascular cuffing with lymphocytic cells. Note the lymphocytes are found and near the gloneruli too.

H & E stain x 100



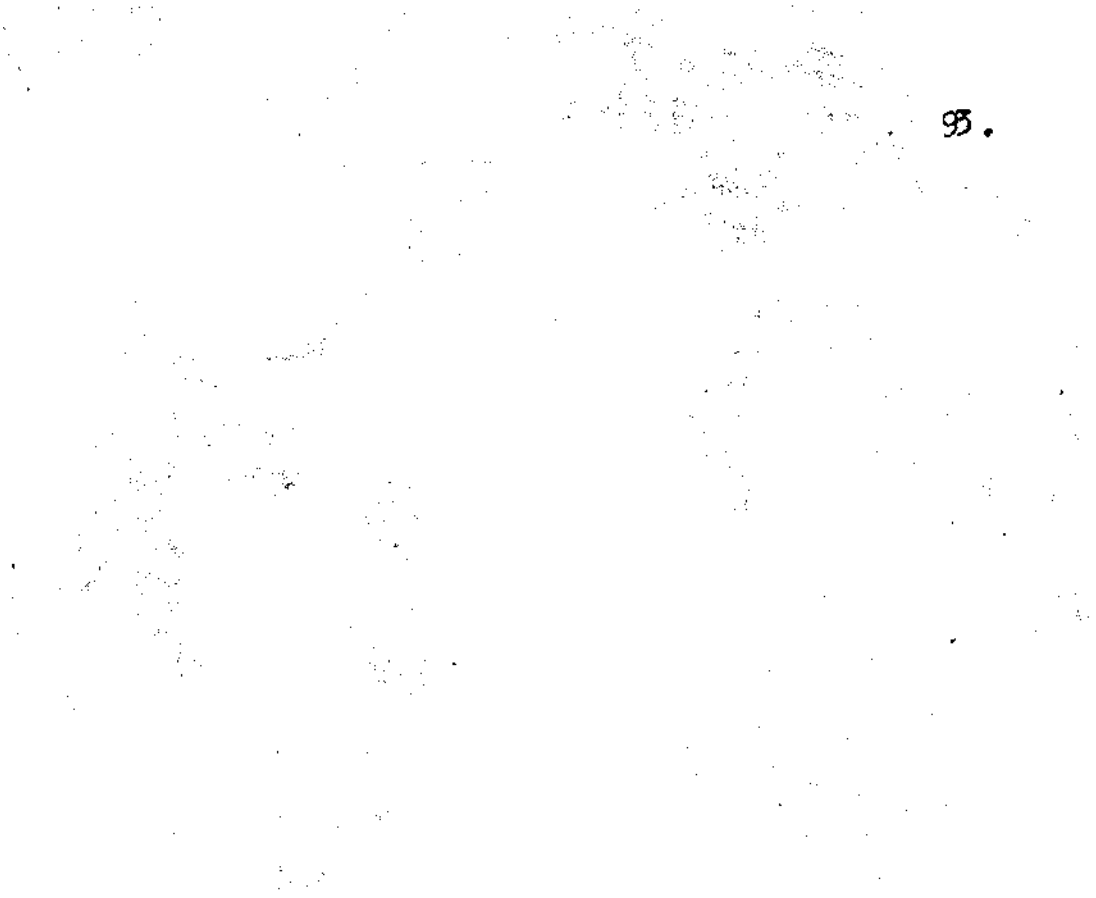


Plate 3. Fig c: Photomicrograph of kidney of mouse infected with Pichinde virus showing perivascular cuffing with lymphocytic cells.

H & E stain x 400.

3.1.9. Histopathology of persistently infected carrier mice:

The extent of virus involvement in the carrier mice was found to be more apparent in the livers and kidneys than in other organs.

Liver:

The livers of four - month - old mice showed lymphoid perivascular cuffing in the portal triad (plate 3.6) Kupffer cells were prominent. The lymphocytic infiltrations in the liver were mixed with macrophages and polymorphs. Pyknotic cells and hydropic degeneration, glycogen and megakaryocytes seemed to be confined in the central vein areas.

Kidneys:

These showed tubular necrosis of distal and some proximal convoluted tubules with eosinophilic and PAS positive casts. The glomerular mesangium was prominent but not hypercellular. Some basement membrance thickening was apparent in a few kidneys. Perivascular cuffing with mononuclear cells was evident (plate 3.7).

3.2.0. Infectious Centre activity of Acutely infected and Persistent carrier mice:

In order to determine if splenic and peripheral blood mononuclear cells (T and B cells) from acutely infected or persistent carrier mice could yield infectious virus in these lymphocytes, lymphocytes from spleens and blood of each group of these mice were taken at intervals after infection and fractionated by nylon wool column or Ficoll-paque method. The fractionated cells (T or B) were tested for infectious centre formation as described earlier in the methods section. The results (table 3. 8) showed significant infectious centres formed by mononuclear cells from acutely infected and persistent carrier mice.

Infectious centre formation was greatest in T cell enriched spleen cells of acutely infected mice than in enriched B-cells. In persistent carrier mice, the T-cell enriched spleen cell had the highest infectious centres formed when compared with those of B-cell enriched population. Peripheral blood mononuclear cells had lower infectious centres than spleen cells (table 3. 8).

These results suggest that effluent non adhering non phagocytic T-cells play vital role in the maintenance of persistent pichinde virus infection as these

cells have been found to contain significant infectious centres. These non adhering mostly T-cells may act as target cells for picninde virus replication thereby resulting in maintenance of the virus.

3.2.1. Effect of T-cell depletion:

To investigate whether or not specific T-cells were responsible for the cytotoxic effects demonstrated in the ^{51}Cr. release assay, Nylon wool column purified cells were treated with anti-theta serum and complement and the cytotoxic effects of the residual cells were checked. The result (table 3.9) showed a considerable reduction in cytotoxicity when compared with untreated control cultures.

This result clearly indicate that both immune mediated damage and viral persistence are mediated to a greater extent by the same set of non adherent, non phagocytic and non immunoglobulin bearing lymphocytes. These cells are characterized as T-cells. It is also possible that natural killer cells (NK) which have somewhat similar morphological characteristics do participate in the cytotoxic effects and establishment of persistent infections.

Table 3.8:- Infectious centre activity in mononuclear cells of CBA mice acutely infected or persistently infected with Pichinde virus.

Cell source	VPFU/10^6 Acutely infected	VPFU/10^6 persistent carrier
Nylon wool column effluent cells (spleen)	92	51
Nylon wool column adherent cells (spleen)	35	22
Nylon wool column adherent cells peripheral blood	4	0
Nylon wool column effluent cells peripheral blood	12	7

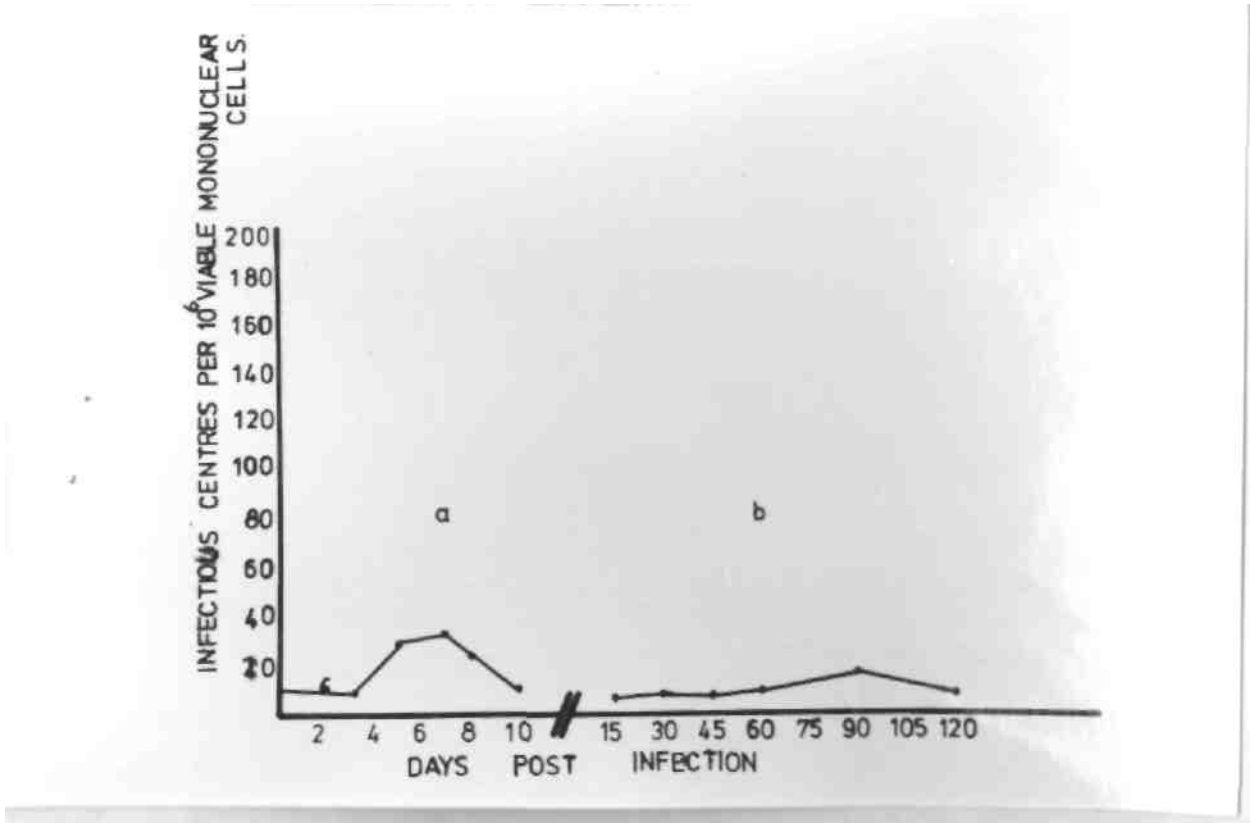

INFECTIOUS CENTRES PER 10^6 VIABLE MONONUCLEAR CELLS.
200
180
160
140
120
100
80
60
40
20
a
b
2 4 6 8 10 15 30 45 60 75 90 105 120
DAYS POST INFECTION

Fig. 3.5: Infectious centre activity of mononuclear cells of CBA mice (a) acutely infected, (b) persistently infected with pichinde virus.

Table 3:9: Effect of T-cell depletion on cytotoxic effect of pichinde virus infected CBA spleen cells on target L-cells

Depletion procedure	% cytotoxicity pichinde infected (mean ± S.E.)	% cytotoxicity uninfected control (mean ± S.E)
Nylon wool column effluent	64.73±17.8	20. ±5.2
Nylon wool column effluent + anti θ + C'	27.86±10.85	16,8±7.2
Nylon wool column adherent cells	25.68±0.68	15.2±4.8
Nylon wool column adherent after carbonyl iron treatment	10.7±4.8	9.5±4.0

3.2.2. Effect of macrophage depletion on cytotoxicity of virus infected targets:

To ascertain whether the in vitro cytotoxic reactions recorded were T-cell mediated or macrophages or B-cells mediated the nylon wool column purified lymphocytes were separated into effluent non adherent mostly T-cells and adherent B-cells and macrophages. Each of these fractions was incubated in plastic petri dishes for one hour at 37°C and the supernates containing non adherent cells were removed and tested for cytotoxicity. The results (Table 3.10) showed no appreciable difference between the cytotoxicity of macrophage depleted and undepleted cells. This suggests that T-cells participate in the in vitro cytotoxicity. Treatment with rabbit anti mouse immunoglobulin plus complement did not alter the cytotoxicity (table 3.11 ' which imply that B cells which have surface immoglobulins do not play a role in the specific cytotoxicity.

Table 3·10. The Effect of Macrophage depletion on Specific Cytotoxicity.

Spleen Cells	Macrophages	% ^{51}Cr Release
Normal (control)	undepleted	20.01 ± 5.7
Normal (control)	depleted	20.41 ± 8.3
Immune T cells	undepleted	54.73 ± 17.8
Immune T cells	depleted	59.98 ± 16.2

Table 3.11: Effect of treatment with rabbit antimouse Immunoglobulin plus Complement on Cytotoxicity.

Spleen Cells	Treatment	% ^{51}Cr Release
Normal (control)	None	26.82 ± 2.3
Immune T cells	None	65.72 ± 9.4
Immune cells	Antimouse + C^1 Immunoglobulin	60.0 ± 8.1
Immune	Normal rabbit serum	62.5 ± 7.1

3.2.3. Blastogenic response of lymphocytes from infected mice:

The *in vitro* examination of blastogenic response of lymphocytes to stimulation with Con A and LPS is one of the most useful *in vitro* parameters of evaluating the *in vivo* cell mediated immunity functions of lymphoid cells. Therefore blastogenesis of T-cell enriched spleen cells from acutely infected mice to Con A mitogen was compared with the enriched cells from their unstimulated infected counter parts. A similar comparison was done with B-cell enriched spleen cells stimulated with LPS mitogen and those unstimulated infected counter parts. The results (Table 3.12) indicated a significant increase in blastogenic response by stimulated lymphocytes of pichinde virus infected animals than from unstimulated infected lymphocyte controls.

Moreover, mitogenic stimulation showed increased levels of infectious center formation when compared to unstimulated cells (table 3.12) though the increase was not significant.

Table 3.12: Effect of mitogenic stimulation of lymphocytes on infectious centre formation:

Treatment	Mitogen	VPFU/10^6	^{3}H Thymidine uptake CPM
Nylon wool column effluent cells	CoNA	47	27,146±2564
Nylon wool column effluent cells	None	43	1,647±127
Nylon wool column adherent cells	CoNA	26	14,196±1023
Nylon wool column adherent cells	None	18	1,315±101
Nylon wool column effluent cells	LPS	17	6,147±857
Nylon wool column effluent cells	None	23	3,526±516
Nylon wool column adherent cells	LPS	31	17,902±1911
Nylon wool column adherent cells	None	27	5,242±1120

3.2.4: Effect of histocompatability difference on cytotoxicity:

In previous studies it was found that compatability at the H-2 gene complex is essential for interaction between sensitized thymus derived T-cells and target cells (McDevitt and Benacerraf, 1969; Vaz and Levine, 1970; Benacerraf and McDevitt, 1972; Kindred and Shrefler, 1972; McDevitt and Bodmer, 1972; Katz et al., 1973· Gardner et al., 1974; Doherty and Zinkernagel and Doherty, 1974, 1975). However in other recent studies (Neustadt et al., 1978) these claims were disputed.

To find out if H-2 differences between effector and target cells would affect cytotoxicity in pichinde virus infection, cytotoxic effects of spleen cells from different inbred strains of mice infected with pichinde virus were assessed against a single target. The results (table 3.13) indicate that H-2 identity between effector T-cells and target L-cells is necessary for maximum specific lysis to occur. This implies that susceptibility of CBA mice to acute lethal disease due to pichinde virus infection is genetically linked to H-2 complex.

Table 3.13: Cytotoxic effects of spleen cells from different strains of mice acutely infected with pichinde virus:

Mouse strain	Haplotype	% Cytotoxicity	
		Pichinde infected Lcells (range)	Uninfected Lcells (range)
CBA	H-2^k	64-86	20-27
BalbC	H-2^d	10-16	10-13
SWR/J	H-2^q	17-24	14-27
A/JAX	H-2^a	17-27	8-25

3.2.5. Mean survival time:

The virus titres and mean survival time of the various inbred strains of mice infected with pichinde virus were examined to ascertain if H-2 differences affect the susceptibility to acute pichinde virus infection. The result (table 3.14) demonstrated that all the mice tested were susceptible although CBA and SWR/J have higher virus titres and lower mean survival time than A/Jax and BalbC. This difference was not statistically significant.

3.2.6. Effect of T-cells depletion:

When anti θ serum + C^1 was used to deplete T-cells fractionated from different inbred strains of mice which were acutely infected with Pichinde virus, there was significant reduction in cytotoxicity in the CBA mice while in other inbred strains, the reduction was less noticeable (table 3.15). Carbonyl iron treatment to remove macrophages also gave significant reduction in cytotoxicity in all the inbred strains (table 3.15).

Table 3.14: Effect of Histocompatability difference on susceptibility to acute pichinde virus infection.

Mouse Strain	Haplotype	$LogLD_{50}$/0.03ml	Mean survival time in days
CBA	$H\text{-}2^k$	5.4 ± 0.4 SEM	9 ± 2.0
SWR/J	$H\text{-}2^q$	5.1 ± 0.2 SEM	9 ± 3.0
A/Jax	$H\text{-}2^a$	4.3 ± 0.4 SEM	11 ± 2.4
BalbC	$H\text{-}2^d$	4.7 ± 0.3 SEM	10 ± 1.7

Table 3.15: Effect of T-cell depletion on cytotoxicity of spleen cells from different inbred mouse strains to pichinde infected Cr-51 labelled target L-cells:

Depletion procedure	% Cytotoxicity (range)			
	CBA	A/J AX	SWR/J	Balbc
Nylon wool effluent T-cells	57-79	28-36	27-47	24-37
Nylon wool effluent T-cells plus anti θ + C'	18-35	22-29	20-33	18-31
Nylon adherent cells	28-39	21-31	18-25	16-28
Nylon wool adherent cells after carbonyl iron treatment	15-21	18-27	15-24	16-19

3.2.7: Role of Humoral Immunity on the Pathogenesis of experimental Pichinde Virus Infection of Mice.

3.2.8: Introduction.

Although circulating antibody has long been regarded as a major defense mechanism against virus infection it is also well-established that target cell destruction mediated through the interaction of antibody with normal lymphoid cells can result in injury to the host (Cerottini and Brunner, 1974). The specificity of the antibody mediated destruction of cells is determined by the antibody bound to the membrane associated antigens and that the effector cell bear receptors for the Fc. fragment of the immunoglobulin molecule. Studies by Greenberg et al. (1975) indicated that the effector cells which mediated antibody dependent cytotoxicity belong to a class of non phagocytic, Fc receptor positive and immunoglobulin negative cells referred to as natural killer (NK) cells.

B cells were found not capable of mediating antibody dependent cytotoxicity as removal of phagocytic cells and passage through a column of sephadex conjugated with purified rabbit anti mouse Fab did not abrogate the cytotoxic activity, also removal of T cells by treatment with anti T cell serum plus complement did not alter the ability of the remaining

fraction to mediate antibody dependent cytotoxicity (Greenberg, 1973). Antibody dependent cytotoxicity is primarily mediated by IgG antibody (Maclennan, 1972 and Perlmann, 1972) and antibody - dependent complement mediated cytotoxicity has been shown to occur in the presence of minute amounts of antibody which arise from B-cells in the spleen (Van Boxel et al., 1972 and Kiessling et al., 1976). Moreover Oldstone et al (1971) showed that LCM virus induced antibody in the presence of complement would lyse LCM virus infected target cells. The possible pathogenetic role of pichinde virus induced antibody was examined by determining the effect of various concentrations of pichinde virus specific antibody plus complement on the lysis of pichinde virus infected target L-cells and comparing the cytotoxicity with uninfected controls. The result (fig.3.6) failed to show significant role of specific pichinde virus antibody and complement against pichinde virus infected target cell.

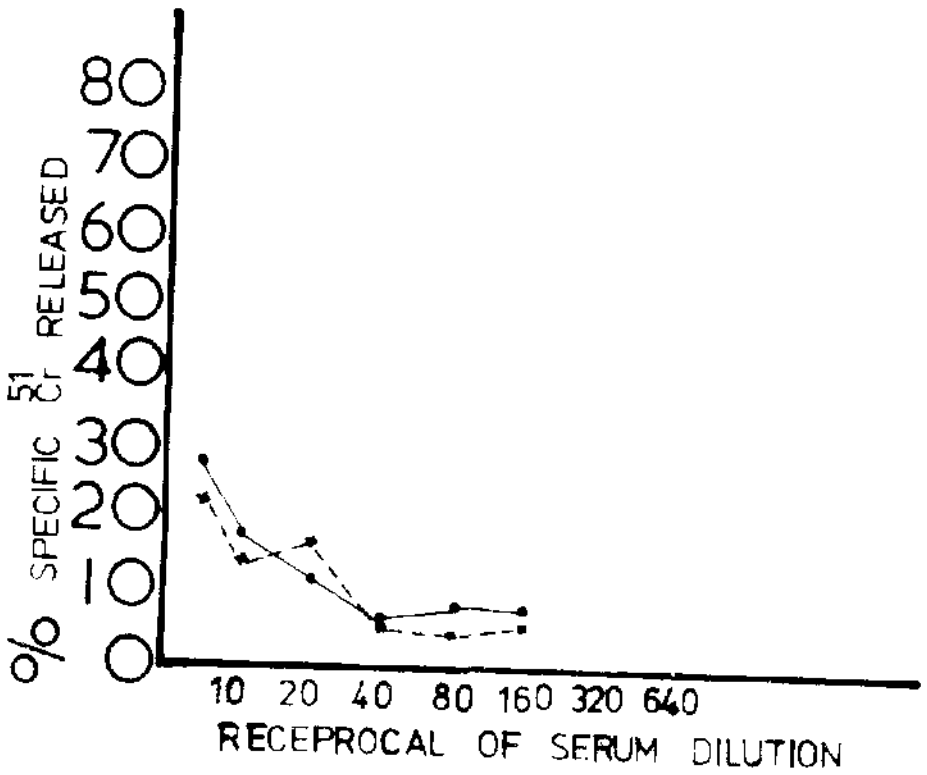

% SPECIFIC ^{51}Cr RELEASED
0
10
20
30
40
50
60
70
80
10 20 40 80 160 320 640
RECEPROCAL OF SERUM DILUTION

Fig. 3. 6: Specific ^{51}Cr released from Pichinde virus infected L cells by anti pichinde serum plus complement: (——) pichinde infected.
(----) control uninfected.

3.2.9. Effect of humoral immunity on pathogenesis of Pichinde virus:

In order to determine the role humoral immunity plays in the pathogenesis or resistance to pichinde virus infection, the development of complement fixing antibody and neutralizing antibody in acutely infected and persistent carrier CBA mice were studied. Sera were taken from acutely infected mice at three days intervals after infection and analysed for complement fixing antibody by using microcomplement fixation test and neutralizing antibody using plaque reduction test in vero cells. The result (table 3.16) failed to show presence of either complement fixing antibody or neutralizing antibody in the sera of acutely infected mice for the first two weeks of infection. From this result it is clear that humoral immunity did not play a vital role in the pathogenesis of acute infection as all acutely infected suckling mice died within two weeks of infection. This meant that cellular immunity which developed within a week of the acute infection might play a vital role in the pathogenesis of acute pichinde virus infection. Significant complement fixing antibody was demonstrable about three weeks after the immunizing dose of acute infection and rising to a high titre at 60 days post infection. This high

complement fixing antibody titre was only apparent in weanling and adult mice that were given immunizing dose of the virus and also in anti thymocyte treated and virus infected suckling mice which were made persistent virus carriers (Table 3.17). Viremia was transient at three days post infection of weanling and adult mice while immunosuppressed persistent carriers continued to demonstrate viremia for up to a period of four months (table 3.18). No neutralizing antibody was detected from either acutely infected or persistent carriers using the plaque reduction test.

From the results of cytotoxicity assays and complement fixing titres of acutely infected mice, it was evident that both suckling and weanling infected mice were adequately sensitized by the virus. The difference in susceptibility between suckling and weanling mice was that the virus titres in organs of suckling mice rose to high levels (fig. 3.3) and cell mediated response developed later and extensive destruction of vital organs by specific T-cells resulting in the death of the suckling mice while in weanling mice virus titres in the blood and organs were significantly lower (table 3.5) and therefore destruction of vital organs was minimal. So the mice survived and were able to clear the virus through humoral immune response.

Table 3.16: Complement fixing and neutralizing antibody titres in acutely infected weanling CBA mice. (9-14 days old CBA inoculated i/p)

Days serum taken post infection	C.F. Titre	Viremia (Log_{10} PFU)	Neutralization antibody titre
3	$\angle$1:4	$10^{3.2}$	$\angle$1:2
6	$\angle$1:4	$10^{2.5}$	$\angle$1:2
9	$\angle$1:4	$10^{1.0}$	$\angle$1:2
12	$\angle$1:4	ND	$\angle$1:2
15	1:8	ND	$\angle$1:2
20	1:32	ND	$\angle$1:2
30	1:64	$\angle 10^{1.0}$	$\angle$1:2
60	1:64	$\angle 10^{1.0}$	$\angle$1:2

ND = Not done.

3.2.8: Effect of antithymocyte serum on the course of pichinde virus infection:

When it was established that cyclophosphamide could not protect suckling CBA mice from acute lethal disease due to pichinde virus infection as was the case with lymphocytic choriomeningitis virus, another experiment was conducted to study the immunosuppressive effect of anti-thymocyte serum on pichinde virus infection. Several reports have already indicated the usefulness of anti-thymocyte serum which affect predominantly cell mediated immunity (James and Anderson, 1967; James et al., 1969; Lance, 1970). Anti lymphocyte serum was found to be effective in converting acute LCM virus infection into persistent carriers Hotchin, 1962; Hirsch, 1967; Hirsch and Murphy, 1968. It was also found to be effective in tacaribe virus infection of mice (Borden et al., 1971).

In this study, group of thirty suckling CBA mice were infected with 10^5 PFU pichinde virus when they were four days old and were each treated with antithymocyte serum (.03ml intraperitoneally) the same day after virus infection and at three days intervals. Thirty suckling CBA mice were similarly treated with normal serum after pichinde virus infection to serve as controls.

Table 3.17: Effect of Antithymocyte Serum on Mortality of Pichinde Infected CBA Mice. (4 days old CBA mice inoculated with pichinde virus i/c)

Age at Infection (days)	Antithymocyte Treatment	% Mortality	Median Survival time (days)
0-1	Normal Serum	100	11
0-1	0.03ml I/P at three days interval	90	25
4	Normal Serum	100	9
4	0.03ml I/P at three days interval	40	> 120
9	Normal Serum	21	> 120
9	0.03ml I/P at three days interval	0	> 120

The results (table 3.17) showed that these persistently infected mice were found to have reduced mortality despite extensive virus replicating in the organs leading to generalised infection of all tissues. In addition these carrier mice continued to have viremia and C.F. antibodies for up to four months post infection when the carrier mice were bled by retro-orbital venous plexus at intervals shown (table 3,18). The infectivity of each serum was assessed using plaque assay in vero cells and by inoculating serial ten fold dilutions of each serum intracerebrally to suckling swiss albino mice. The result (table 3.19) demonstrate infectivity of some of the sera. To ascertain that this effect was not due to toxic manifestations of the sera but was due to infectious virus complexed with antibody, these sera were re-examined by mixing equal volumes of each serum with antimouse gamma globulin and the mixture incubated for one hour at 37°C and then at 4°C over night. The sera were centrifuged and supernatant inoculated into suckling swiss albino mice. Control mice either inoculated with a mixture of anti mouse gamma globulin and PBS or inoculated with the virus alone were included. From the results (table 3.20) it was evident that after precipitation with antimouse gamma globulin the infectivities of the sera were reduced considerably.

Table 3.18: Complement fixing antibody titre and viremia in persistently infected pichinde virus carrier CBA mice: (4 days old CBA mice inoculated with pichinde i/c and antithymoyte serum given at 3 days intervals i/p).

Days serum taken post infection	CF Titer	Viremia PFU/ml	Neutralizing Antibody (plaque reduction)
3	<1:4	$10^{3.5}$	-ve
5	<1:4	$10^{4.2}$	-ve
10	1:4	$10^{1.3}$	-ve
15	1:8	$10^{1.3}$	-ve
20	1:8	ND	-ve
25	1:8	ND	-ve
30	1:32	ND	-ve
35	1:32	$10^{2.5}$	-ve
40	1:64	$10^{2.2}$	-ve
50	1:64	$10^{2.2}$	ND
60	1:64	$10^{2.0}$	ND
90	1:64	$10^{1.1}$	ND
120	1:32	$10^{1.3}$	ND

+ve control asciticfluid titre = 1:1024

+ve control rabbit antipichinde antibody tibre = 1:128

ND = not done.

Table 3.19: Infectivity of serum from CBA mice persistently infected with pichinde virus.

Inoculum	No. of mice Inoculated	No. of mice dead	% mortality
Pichinde virus	8	8	100
PBS	8	0	0
Neat serum from carrier mice	8	8	100
Serum from pichinde carrier mouse diluted one in ten	8	8	100
Immune ascitic fluid + Pichinde virus	8	8	100

Table 3.20: Effect of Treatment of persistently infected CBA serum with Antimouse gamma globulin on infectivity:

Inoculum	No. of mice inoculated	No. of mice dead	% Mortality
Pichinde virus diluted $\frac{1}{4}$	10	10	100
Pichinde carrier serum diluted 1/10	10	10	100
Pichinde carrier after ppt with antimouse gamma globulin	10	4	40
Antimouse gamma globulin + PBS	10	0	0
Pichinde carrier serum diluted 1:100	10	9	90
Pichinde carrier serum diluted 1:1000	10	4	40
Pichinde carrier serum diluted 10^4	10	2	20

3.2.9: Effect of Adoptive Transfer of Immune Spleen cells to Persistent Pichinde Carrier CBA Mice.

When fractionated early immune T-cells (day 9 post Infection) from acutely infected CBA mice were injected intraperitoneally (10^7 cells) to persistent pichinde carrier mice, all the carrier mice died with convulsions typical of lethal pichinde virus infection (table 3.21). Fractionated secondary immune T-cells taken from weanling CBA mice thirty days after immunizing dose of the virus also killed all the persistent carrier mice while mortality of mice injected with normal fractionated T-cells from uninfected mice was less (table 3.21). This result clearly shows that adoptive transfer of immune spleen T-cells can convert persistent carriership into lethal disease rather than eliminating viremia.

Table 3.21: Effect of Adoptive transfer of Immune Syngeneic Spleen cells to persistently Infected CBA mice.

Donour cells	Mean ^{51}Cr Release	Dead/ Total	% Mortality
Primary Immune Spleen cells (9 days)	70.61	20/20	100
Secondary Immune Spleen cells (30 days)	10.90	20/20	100
Normal Spleen cells	24.88	4/20	20

3.3.0: The role of Immune complexes in the pathogenesis of Pichinde virus in CBA mice:

As mentioned earlier in the literature review immune complexes have been found to be important in the pathogenesis of a number of diseases such as hepatitis virus infection, lactic dehydrogenase virus infection and among the arenaviruses lymphocytic choriomeningitis virus infection of mice was found to result in immune complex formation leading to tissue injury.

The result of the infectivity assays (tables 3.18 and 3.19) did not prove conclusively that these persistently infected mice contained virus antibody complexes circulating in their blood. So further examination of the sera of these animals were undertaken using a relatively sensitive iodine labelled C1q binding assay developed by Zubler et al (1976) and modified by June et al (1979) when testing mouse sera for immune complexes. The result (fig. 3.8) showed presence of immune complexes by C1q binding assay. The immunoglobulin levels in the sera of a few mice with high immune complex levels were estimated (fig. 3.10) which showed increased IgG levels in the carrier mice compared to uninfected controls. The immune complexes were mostly of the IgG class as determinated by radial immuno diffusion.

It was further observed (fig. 3.9) that the level of C1q binding material (Immune complexes) significantly increases as the duration of persistency becomes longer. This suggests that in mice persistently infected with pichinde virus, circulating virus - antibody immune complexes continue to form and subsequently deposit in the kidneys, and arteries resulting in virus - antibody immune complex disease.

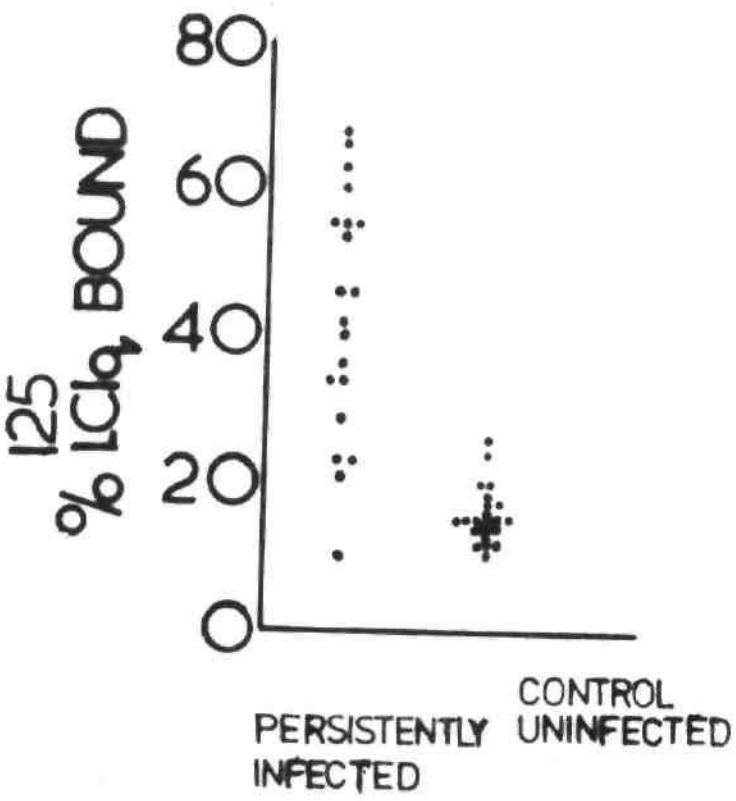

125 % $I^{125}C1q$ BOUND
80
60
40
20
0
PERSISTENTLY INFECTED
CONTROL UNINFECTED

Fig. 3.8: C1q binding activity in sera of individual CBA mice persistently infected with pichinde virus compared to those age matched uninfected controls. Each point represents data from one mouse.

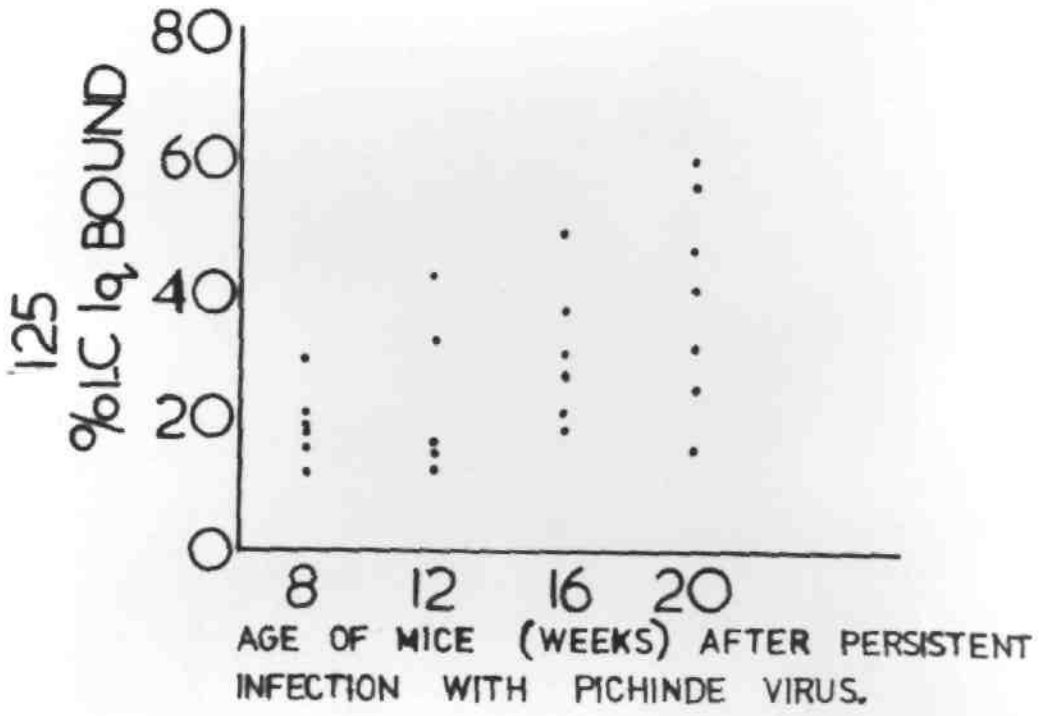

125
%I.C Iq BOUND
80
60
40
20
0
8
12
16
20
AGE OF MICE (WEEKS) AFTER PERSISTENT INFECTION WITH PICHINDE VIRUS.

Fig. 3.9 : C1q binding activity in sera of individual mice persistently infected with Kichinde virus. Each point represents data from one individual mouse.

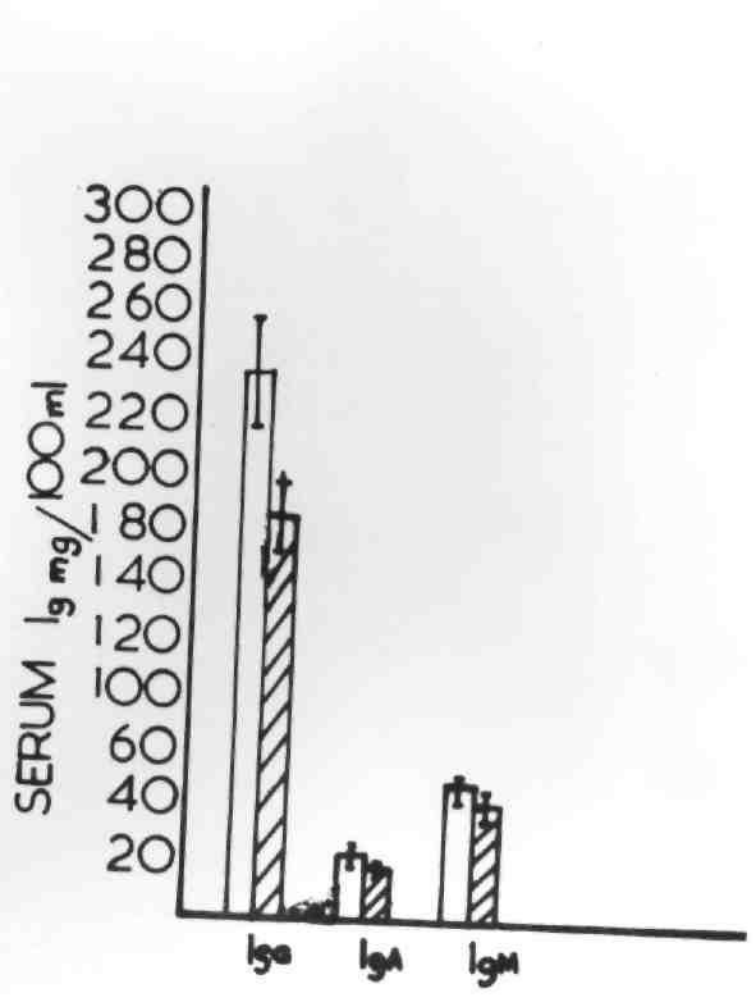

SERUM Ig mg/100ml
300
280
260
240
220
200
180
140
120
100
60
40
20
IgG
IgA
IgM

Fig. 3.10: Mean serum Immunoglobulin levels of persistent pichinde carrier mice (open bars) compared to uninfected control mice of the same age group (hatched bars). Mean value of seven mice ± SEM.

CHAPTER FOUR

4.1.0. DISCUSSIONS

Although in most circumstances immunological reactions play an important role in recovery from viral infections as has been shown when sensitized lymphocytes from infected host recognized virus specific antigens on the surfaces of infected cells and destroy these cells or when lymphocytes in response to antigenic stimulation liberate interferon or other lymphokines to protect the host against the invading agent, in some circumstances immunological reactions initiate or aggravate tissue damage which may be so severe as to kill the infected host.

Numerous studies with lymphocytic choriomeningitis virus, the prototype of the arenaviruses, have shown that experimental intracerebral infection of adult mice leads to fatal immunopathologic disease of the central nervous system while infection of neonatal mice leads to chronic persistent carrier state which also ultimately develops into fatal immunopathologic disease due to deposition of virus - antivirus immune complexes on the glomeruli of the kidney, arteries and choroid plexus of the brain.

4.1.1. Cell mediated immunity in the pathogenesis of Pichinde virus infection:

In this study the role of cell-mediated immune response in the pathogenesis of experimental acute pichinde virus infection of CBA mice was demonstrated using an *in vitro* model of cell-mediated immune response that involves quantitative *in vitro* destruction of virus

infected Cr-51 labelled target cells by cytotoxic thymus derived lymphocytes generated during acute pichinde virus infection. This method had earlier been successfully used as a reliable measure of cell mediated immune response in viral infection (Steele et al., 1973 and Bellanti et al., 1976). Thymus derived lymphocytes obtained from spleens of mice acutely infected with pichinde virus eight to nine days previously showed strong specific lysis on target cells as could be shown by the alveolar septal thickening of the lung resulting in the narrowing of the lumina and hyalinization of some vasculature. In the liver lymphoid perivascular cuffing in the portal triad is also evident. Further more, liver showed lymphocytic infiltration mixed with macrophages and polymorphs and there were pycnotic cells and hydropic degeneration (Plates 3.5, and 3.6) while T. lymphocytes obtained from normal uninfected animals or those infected with vasicular stomatitis virus did not show any significant lysis on pichinde virus infected syngeneic target cells. The brief time course for the cell-mediated immune response which developed during the acute infection coincided with the period of viremia (table 3.8) and intense histopathologic response with infiltration of mononuclear cells and massive necrosis in the liver, spleen, kidneys and brain (plates 3.1, 3.2, 3.3, 3.4 respectively). In experimental pichinde virus infection of CBA mice the development of cell-mediated immune response (as measured

by in vitro cytotoxic assay) was positively correlated with the degree of histopathologic response as there was severe hepatitis of the infected mice (plates 3.1 and 3.6). This hepatitis contributed greatly to the clinical signs and fatal outcome of the infection. The histopathological picture of the infection has similarities with these reported in fatal case, of human lassa fever (Winn and Walker, 1975). Similar alterations have also been reported in experimental lassa fever of monkeys (Walker, Wulff and Murphy, 1975) and experimental Bolivian haemorrhagic fever in rhesus monkeys (Terrell et al., 1973). When these mice were immunosuppressed following infection with the virus, they were found to be free of these histopathologic alterations in their lymphoid organs except for the spleens of these mice that showed diffuse and follicular depletion of lymphocytes. Similar findings were reported in mice infected with amapari and tamiami viruses following thymectomy (Besuchio et al., 1973). It was clear from this result that immunosuppression resulted in diminished clinical expression of the acute infection while passive transfer of immune lymphocytes from syngeneic donors to immunosuppressed infected mice resulted in increased severity of the infection.

Evidence that T lymphocytes were responsible for the acute immunopathologic manifestations of the disease was shown by the fact that cytotoxic activity in vitro could be reduced considerably by prior treatment of the fractionated immune T-cells obtained from the spleens of acutely infected mice with anti theta serum plus complement (table 3.9). The specificity of the T-cells was also established by the failure of the T cells to lyse normal uninfected syngeneic target cells or target cells infected with unrelated virus. Depletion of macrophages and B cells did not significantly reduce the cytotoxic activity (table 3.10), neither was treatment with anti mouse immunoglobulin plus complement (table 3.11). In vivo treatment with anti mouse thymocyte serum resulted in significant reduction in lethality of acute infection depending on the day after infection at which the treatment was started. (table 3.17) which further confirmed that the acute lethal disease was due mainly to cellular immune response against pichinde virus infection.

During this period of intense cellular immune response (8 - 9 days post infection) no trace of humoral immune response was shown as there was no detectable complement fixing or neutralizing antibodies (table 3.18).

In contrast to studies with lymphocytic choriomeningitis virus which showed that prior treatment with cyclophosphamide resulted in the elimination of the

acute immunopathologic disease and the establishment of persistent lymphocytic choriomeningitis virus carrier state cyclophosphamide was not found to be an effective immunosuppressant in pichinde virus infection as the drug did not alter the susceptibility of the mice to acute pichinde virus infection (table 3.4). Only repeated intraperitoneal injection of antithymocyte serum was effective in reducing the acute lethal disease due to pichinde virus infection. This might be due to the possibility that cyclophosphamide acts mainly to eliminate the B cell compartment of the immune response as suggested by Turk and Poulter (1972) and Stockman et al., (1972), while antithymocyte serum and antilymphocyte globulins specifically eliminate T cell compartments (James and Anderson, 1967; James et al., 1969 and Lance, 1970).

Histopathological results have in fact shown that cyclophosphamide treatment depleted mainly the white pulp areas of the spleen which have been reported to constitute a major B cells population.

4.1.2. Histocompatability Difference:

In this study a major histocompatability complex restriction has been demonstrated for T cell mediated cytotoxicity of pichinde virus infected target cells (table 3.15). The results showed that T lymphocytes removed from spleens of CBA mice acutely infected with pichinde virus could lyse pichinde virus infected

target L929 cells which express the same haplotype as the effector cells (both target L cells and the CBA effector T cells express the same H-2^k specificity). Other inbred mouse strains which express different H-2 haplotypes from that of target L cells gave significantly lower lysis (table 3.15). From the inbred strains of mice studied in this work, H-2 differences did not affect susceptibility to acute pichinde virus infection as all the inbred strains of mice tested had similar susceptibility and mean survival times (table 3.16). It might be possible that other inbred strains of mice with different H-2 haplotypes which have not been included in the study might show differences in susceptibility.

4.1.3. Immune Complexes In Pathogenesis of Pichinde Virus:

Circulating antibody has been regarded as a major defense mechanism against virus infections as demonstrated when virus is exposed to excess antibody, the infectivity of the virus is neutralized. In viruses that are cytopathogenic humoral immune response protects the animal from the lethal virus infections. However, some viruses such as the arenaviruses produce little or no tissue damage to the host unless accompanied by the immune response where immunopathological reactions may be so severe as to kill the infected animal. Evidence that antiviral antibody plus complement could injure virus infected cells was reported by Brier et al (1971)

who showed that in the absence of specific antiviral antibody plus complement no such injury took place. Virus - antibody complexes have been associated with inflammatory diseases in animals (Oldstone and Dixon, 1971) as the presence of virus - antibody complexes in the circulation can lead to symptoms of serum sickness, glomerulonephritis, vasculitis, arthritis etc.

Results from this study have shown that humoral immune response did not play an important role in the immunopathologic manifestations of lethal disease due to acute pichinde virus infection as anti viral antibody did not appear at the time of acute lethal disease ( ). As the virus is relatively non cytocidal the acute lethal disease was not due to cytopathogenicity of the virus but was a result of massive cell-mediated immune response against the viral antigen which caused the acute lethal disease. However, in the persistent carrier mice, there was evidence that immunopathologic lesions in the livers and kidneys were the result of soluble virus anti-virus antibody complexes which deposited in the renal glomeruli and other organs. Histopathologic lessions consisting of fibrinoid necrosis and perivascular inflammations (plates 3.7 ,) which are associated with the persistent carrier mice might be due to interactions between the virus-antivirus antibody deposited in these organs. This could not be confirmed by immunofluorescence studies.

It was also noted that the sera of all the persistently infected mice continued to have infectious virus complexed with antibody and the infectivities of these sera could not be neutralized by antiviral antibody (table 3.19). The infectivities of the sera were neutralized only by further addition of antimouse gamma globulin (table 3.20).

Circulating immune complexes were sought in the sera of these mice using radio labelled C1q binding method (June et al., 1979). It was quite evident that most of the persistent carrier mice had significantly high circulating immune complexes in their sera when compared with normal uninfected controls of the same age group (fig. 3.7). The percent of trichloro acetic acid precipitable C1q radioactive counts in duplicate test sera was compared with negative and positive C1q binding control sera. It was found that most of the CBA mice persistently infected with pichinde virus for upto five months duration had a high level of circulating immune complexes when compared to uninfected controls (fig. 3.17). There was positive correlation between C1q binding activity and immunoglobulin levels as mice with increased levels of C1q binding activity also showed increased immunoglobulin levels particularly IgG (fig. 3.9) in the sera.

Although histopathological observations have indicated that the persistently infected mice have glomerulonephritis of low severity as there was mild renal disease in all the animals observed, the intensity of glomerulonephritis

might increase when these carrier mice were kept for a longer period of time say one year. It will be of interest in future to determine if there is genetic control over the formation and deposition of immune complexes by various inbred strains of mice infected with pichinde virus as it has been reported that antibody responses to various antigens are controlled by genes mapping in theIr-region of the H-2 complex (Benacerraf and McDevitt, 1972; Klein, 1975 and McDevitt et al., 1972). There is also a need to determine the binding affinities of the antibody produced from various inbred strains of mice infected with pichinde virus and determine the role the binding affinity of the amtibody produced on the formation and deposition of immune complexes and subsequent tissue injury. Steward et al. (1975), Petty and Steward (1977) have shown that low affinity antibodies resulted in production of immune complex disease. There is also a need to determine if sex of the animal can influence the formation of immune complexes. This work did not take this factor into consideration because limited number of mice available did not permit adequate pooling of these mice according to sex. Many studies have shown that female animals generally produce more immune complexes and are more nephritis prone than their male counterparts of the same age (Steward et al, 1975, Petty and steward 1977). In addition it would be of interest to determine the specificity of the C1q binding material. Whether all the

structural or non structural polypeptides of the pichinde virus act as components of the immune complex. This work did not determine the specificity as the recovery of material in the C1q bound complex was insufficient to allow peptide analysis to ascertain what type(s) of pichinde polypeptides were contained in the complexes. Recent studies by Oldstone et al. (1980) on lymphocytic choriomeningitis induced immune complex diseases have demonstrated that anti lymphocytic choriomeningitis antibody in the complex reacted specifically against the three main LCM virus structural polypeptides - nucleoprotein and the two glyco peptides.

It is suggested by Welsh and Oldstone (1977) that Lymphocytic choriomenininingitis virus escapes immunological surveillance during life long persistent infection of mice by producing defective interfering (D.I) virus which inhibit surface expression of viral antigens and subsequently preventing effective killing of infected target cell by either cytotoxic T cell and by antibody plus complement but nevertheless producing sufficient antigen to elicit strong immune response against all the LCM virus structural proteins.

Evidence from this study has shown that pichinde virus replicates in macrophages and T cells and this has also been shown in Lymphocytic choriomeningitis virus (Nims and Subrahmanyan, 1966), Tamiami virus

(Murphy et al., 1976). It is possible that pichinde virus persistence is achieved by the virus replicating in the mononuclear cell and then be readily transmitted to other organs. The low level of infectious centres observed might not represent the maximum number of virus infected cells, it probably represented only by those infected cells that were replicating the virus.

Pichinde virus persistence could also be maintained in mice in the form of virus - antibody complexes circulating in the presence of suppressor cells that inhibit production of cytotoxic offector cells which are involved in virus clearance or clinical disease. Recent evidence by Chan, Clark and Rawls (1983) has shown that pichinde virus infection of MHA hamsters induced suppressor cell production which could inhibit foot pad swelling response and prevent lethal disease.

Another possibility of pichinde virus persistence in a form of virus antivirus immune complexes could be that the virus replicates in macrophoges - T cells which would result in aberrant effector cells involved in the virus elimination. It was reported by Casali et al (1981) that glycoproteins from lymphocytic choriomeningitis and from measles virus when inserted into artificial membranes or in soluble form, could induce significant non specific cell mediated cytotoxicity in human peripheral blood lymphocytes. Since pichinde virus has been shown to replicate in macrophages, it is possible that virus replication in the macrophages (which are involved in antigen presentation) with subsequent expression of the viral glycoprotein on the surface of infected cells. The glycoprotein and cell surface antigen complex is recognized specifically by cells involved in the induction of suppressor cells leading to increase in production of suppressor cells which consequently interfere with cells involved in virus clearance.

CHAPTER FIVE

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