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Arenaviruses

by

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Preface

The recent explosive progress in medical virology resulted to a large extent from rapid advances in recombinant DNA techniques, in molecular and cell biology, in protein and nucleic acid chemistry, the use of monoclonal antibodies, progress in antiviral research and the application of modern technology to fundamental biological processes. These advances in knowledge of structural and biochemical components of viruses and mode of viral replication, understanding of cell pathology and immunopathogenesis have many health implications which include rapid, precise and specific diagnosis, epidemiology of viral infections, and the development of new types of vaccines. Strengthening of the links between the laboratory, field work and the clinician is essential for implementing the strategy of the World Health Organisation's programme of health for all by the year 2000. Infectious diseases – and viral infections – are still responsible for most of the problems encountered daily in primary health care in many parts of the world.

The number of new journals introduced during the last decade and the avalanche of information have made it virtually impossible for microbiologists, epidemiologists, pathologists, scientists and physicians to keep abreast of the scientific literature. We recognise the age of rapid dissemination of information retrieval by computerised systems, the ever increasing published proceedings of meetings and symposia, abstract and rapid literature surveys, but it is secondary literature in the form of review series which has become useful as sources of information. However, their value depends intrinsically on authoritative and critical evaluation of the original data by experts in particular topics. The series on Perspectives in Medical Virology was conceived after many discussions with teachers of postgraduate students, research workers, medical virologists, students and many colleagues.

A number of distinguished practising virologists were then invited to assemble the important information available and to integrate research at the basic level with clinical practice in selected subjects for the new series. We hope that in this way the series will promote dissemination of information, useful discussion and exchange of ideas and stimulate further interest and research in medical virology.

Arie J. Zuckerman

To

Liane
Wendy
and
Christina

Foreword

The arenaviruses are a unique virological group containing several attractive virus-host systems for experimental study. At the same time, their relatively small number allows useful comparisons to be made both at the molecular and whole animal levels. For example, persistent lymphocytic choriomeningitis virus infection in mice has been the subject of intense interest among microbiologists and pathologists alike since its chance discovery almost half a century ago. Yet this virus differs in several important aspects from other more recently recognized arenaviruses in terms of disease patterns. These newly characterized agents retain many of the biochemical and immunochemical features of lymphocytic choriomeningitis virus which continues to be regarded as the prototype member of the virus family arenaviridae. Certain of these recently recognized arenaviruses have achieved notoriety as aetiological agents of severe haemorrhagic disease in man and are recognized by the medical virologist as rare, but important, 'exotic' viruses. Possible introduction of Lassa fever virus, for example into temperate climates from endemic areas is a matter of great concern to public health authorities. However, it is not widely recognized that other members in the group do not cause significant human illness, although appear remarkably similar in all other respects, and several are closely related in chemical properties to those pathogenic for man. Furthermore, it is now clear that human infection due to Lassa virus is more widespread in endemic areas than previously supposed. Many questions regarding the epidemiology of such infections still remain to be clarified, and significant new arenavirus pathogens of man have yet to be recognized. It is noteworthy, for example, that there

are no recorded isolates from the Far East but this may simply reflect an absence of serious investigation for these viruses in the Asiatic regions.

The immunopathological mechanisms which distinguish arenaviruses, their infections and inter-relationships with natural rodent hosts are important questions which remain to be resolved. The combined tools of immunology, biochemistry and pathology are only now being brought together to form a framework for understanding this group and to provide answers with wider implications for the general study of virus-host relationships. Such studies extend also to the molecular level; arenaviruses are unique in apparently being the only viruses consistently associated with host cell ribosomes in a manner that is yet to be defined. These questions are the themes of this monograph, in which I have attempted to explore the nature and properties of the arenaviridae family in a way that I hope will stimulate further experimentation in as yet unexplored areas of arenavirus research.

In the preparation of this review, I have been encouraged throughout by Professor A.J. Zuckerman, the general editor of this series, and Professor D.I.H. Simpson who first introduced me to arenaviruses. In addition, I am indebted to many others who have, and continue to provide, a source of never ending enthusiasm and ideas. Among these are Paul Young, Jill Dixon, Lisa Allison, Mark Salter and George Mann. My special thanks also go to Dr Michael Buchmeier and Hanna Lewicki, Scripps Clinic and Research Foundation, La Jolla, California with whom I spent a very enjoyable and fruitful year during which the early drafts of this monograph were prepared. Also Drs Karl Johnson, Fred Murphy, Julio Maiztegui and Mercedes Weissenbacher for sharing with me their considerable experience with these pathogens. Much of the typescript has been prepared by Mrs Sandy Giles, Miss Tanya Gidman, Mrs Rose Howard and Mrs Anne Sprawson to whom I owe enormous debt for unfailing cheerfulness at all times. Finally, I wish to acknowledge the patience and support of my wife Liane, and Wendy and Christina, who unknowingly have made their own small contributions. In the last resort, however, I take full responsibility for any errors or omissions.

C.R. Howard
London, March, 1985

CHAPTER 1

Introduction

During the past half century many areas and natural habitats in tropical and subtropical regions of the world have diminished or been radically changed as man makes efforts to increase agricultural production and energy supplies sufficient for his needs. Such progress has occurred perhaps most dramatically in the developing world where the fragile balance within ecosystems has been altered, perhaps irreversibly. The result has been that the medical profession has become increasingly aware of zoonotic infections in human populations as a result of shifting population dynamics and behaviour of animals persistently infected with viruses sharing the same habitat. Johnson (1982) has pointed out that the increasing discovery of pathogens of frightening virulence may sadly indicate the expansion generally of virus infections within such animal populations. The resulting problems of diagnosis, treatment and eventual immunoprophylaxis will take every effort on the part of the medical community to contain. The study of arenaviruses is therefore but one facet of the problem of studying man's effect on his habitat whereby viruses are present as control effectors. It is necessary to accept on this premise that other related or unrelated pathogens will shortly come to light upon which our experience and knowledge from the study of arenaviruses may be applied.

1.1. Historical perspective

The identification and study of arenaviruses has followed essentially two

parallel pathways. On the one hand, lymphocytic choriomeningitis virus (LCM) has been studied for nearly half a century as a model for understanding the interaction between host and virus as manifested by various immunopathological responses measured in laboratory animals. Although capable of causing meningitis and encephalomyelitis in man, infections outside the laboratory are rare. LCM virus was discovered by Armstrong and Lillie (1934) during intracerebral passage in monkeys of human tissue isolated at autopsy from an apparent case of St. Louis encephalitis. Although the primates developed the typical signs of the latter disease over a number of passages, the presence of an additional agent was suspected when material was passaged into animals known to have been previously exposed to the agent of St Louis encephalitis. The symptoms differed in the immune monkeys and a virus termed as experimental lymphocytic choriomeningitis was subsequently reisolated by intracerebral inoculation of mice. Whether the virus was concurrently present with the virus of St. Louis encephalitis in the original patient or was introduced independently into the monkey colony was never resolved. However, the similarity of this infection in monkeys due to LCM virus and previously recognized aseptic meningitis in man stimulated Rivers and Scott (1935) to attempt isolations from patients with acute meningitis. LCM virus was isolated from five patients and the causal relationship between this agent and human aseptic meningitis further confirmed by the demonstration of positive seroconversion to the virus in a number of patients (Rivers and Scott, 1936b; Scott and Rivers, 1936; Armstrong and Dickens, 1935). In parallel with these findings, Traub (1935a) reported that a colony of albino mice was infected with a virus which produced a similar illness to that reported by Armstrong and Lillie. These strains of LCM virus were rapidly established as being related serologically (Armstrong and Dickens, 1935; Rivers and Scott, 1936a; Traub, 1935a), and the link between human LCM infection and naturally infected house mice established soon after (Armstrong and Sweet, 1939). The demonstration of LCM virus in wild-caught *Mus musculus* by numerous investigators leaves no doubt that this peridomestic rodent is the natural reservoir of this virus. The apparent ubiquity of this virus combined with frequent interchange between laboratories has resulted in a diversity of isolates being studied at various times. Of these many can be traced back to original documented isolations, as summarized in Table 1.2.

The term 'viral haemorrhagic fever' has been loosely used during the past 30 years to describe a clinical syndrome common to a number of diseases, including several arising from arenavirus infection. In contrast

TABLE 1.1.
Nomenclature of arenaviruses and date of isolation

Virus	Origin of name	Year	Reference
Old World			
Lymphocytic choriomeningitis	Disease	1933	Armstrong and Lillie (1934)
Lassa	Township, east Nigeria	1970	Frame et al. (1970)
Mopeia	Township, Mozambique	1977	Wulff et al. (1977)
Mobala	Rodent host, Central African Republic	1980	Gonzalez et al. (1983)
New World			
Junin	Township, Argentina	1957	Parodi et al. (1958)
Tacaribe	Ancient tribal group, Trinidad	1956	Downs et al. (1963)
Machupo	Small river, Beni region, Bolivia	1962	Johnson et al. (1966)
Amapari	River, Amapa Territory, Brazil	1964	Pinheiro et al. (1966)
Tamiami	Florida highway, U.S.A.	1963	Calisher et al. (1970)
Latino	Township, eastern Bolivia	1965	Webb et al. (1973)
Parana	River border of Paraquay	1965	Webb et al. (1970)
Pichinde	Upland area, Columbia	1970	Trapido and San Martin (1971)
Flexal	Amazonian tributary, Brazil	1975	Pinheiro et al. (1977)

to many pathogens in this disease category arenaviruses are not transmitted by arthropod vectors but invariably occur as natural infections of various rodent species (Table 1.1). For three decades yellow fever was the only epidemic virus disease known to result in haemorrhagic illness among a proportion of individuals infected. However, many instances of haemorrhagic fevers of both arbovirus and non-arbovirus origin have been recognized with an increasing frequency since the interval between the two world wars. Most notable of those which does not apparently spread by an arthropod vector is the so-called haemorrhagic fever with renal syndrome, which represents a major public health problem in the Far East and across the northern hemisphere. In contrast, arenavirus haemorrhagic fevers occur below the Equator, as far as is presently

TABLE 1.2.
Commonly studied strains and isolates of arenaviruses pathogenic for man

Virus	Strain	Origin	Reference
LCM	Armstrong (CA-1371)	Human	Rowe et al. (1963)
	E-350	Human	Rivers and Scott (1936a)
	Traub	Mouse	Volkert (1962)
	UBC	Human	Hotchin and Wiegand (1961b)
	Pasteur	Human	Riviere et al. (1977)
Machupo	Carvallo	Human	Johnson et al. (1965b)
Junin	XJ	Human	Parodi et al. (1958)
	MC-2	Rodent	Grau et al. (1981)
Lassa	LP	Human	Buckley and Casals (1970)

known. The first case of a new haemorrhagic disease from South America was reported in 1955 near the town of Junin in the Argentine pampas (Arribalzaga, 1955). Cases have continued to occur in the autumn of each year, and almost certainly this disease was recognized inside the endemic area for many years preceding initial reports in the scientific literature. At the present time, the agent of Argentine haemorrhagic fever is perhaps the most accessible human arenavirus for intensive study: although highly pathogenic for man, the lower viraemia levels make patient care somewhat easier than, for example, many patients with Lassa fever. Also, excellent records and careful studies regarding the course of Argentine haemorrhagic fever are available and much is known concerning the effect of Junin virus on the haemopoietic and other systems in infected individuals and experimental animals. The dual approach of developing an attenuated vaccine and investigating the cross-protection in animals using related viruses increases the likelihood that this arenavirus will be the first arenavirus to be controlled using conventional approaches in immunoprophylaxis. A clinical similarity was noted in 1962 between Argentine haemorrhagic fever and a new disease in Bolivia brought to the attention of U.S. virologists working at the Middle America Research Unit, Panama. This syndrome was first recognized a few years previously among rural workers from the north-eastern plateau of the country and was at first suspected as being epidemic typhus. However, the causative agent was identified as possessing the properties of a virus after successful isolation from patients and peridomestic rodents during an urban epidemic around the town of San Joaquin (Johnson et al., 1965b). Study of

the agent of Bolivian haemorrhagic fever, termed Machupo virus, was instrumental in the establishment of a relationship between the South American haemorrhagic fevers and LCM virus.

The initial isolation of Machupo virus was made in newborn hamsters inoculated with a splenic extract from a fatal case of Bolivian haemorrhagic fever. This isolate, designated the Carvallo strain, was examined further by Webb who first indicated that some similarities existed between the ability of LCM to persist in mice inoculated shortly after birth and chronic Machupo infection in surviving hamsters (Webb, 1965). Although no serological cross-reaction was found between the two viruses by complement fixation, Murphy et al. (1969) made the critical observation that these viruses had a remarkably similar and distinctive morphology when examined by thin section electron microscopy. Included in these studies was Tacaribe virus, isolated some years before during a study of rabies infection among the bat population of Trinidad. Although no evidence of human pathology due to Tacaribe virus has ever been found, a serological relationship was demonstrated with Junin virus isolated some 3000 miles distant. Almost certainly the few virus particles seen by Bergold and colleagues of Tacaribe infected cells were the same particles subsequently seen by Murphy et al. (1969) in lymphoid tissue from infected rodents. As Tacaribe virus was the first of these agents to be isolated, the three viruses from South America were collectively referred to as belonging to the Tacaribe complex of the newly-defined group of aren*o*viruses. After the initial proposal of this name (Murphy et al., 1969), the term was modified slightly to aren*a*viruses to avoid any confusion with adenoviruses.

Although the first isolates from South America were at first erroneously designated as newly defined arboviruses, there is no evidence to implicate arthropod transmission of any arenavirus. Nevertheless, similar techniques of isolation, necessity for trapping small mammals and the predominant interests of early investigators, have meant that the majority of arenaviruses have been isolated by workers specialised in this field. During the course of further investigations into the distribution of Machupo virus among natural rodent populations, virologists at the Middle America Research Unit isolated two further arenaviruses from Bolivia and Paraguay, respectively Latino and Parana (see Table 1.1). Interestingly, Latino would not have been discovered but for the use of suckling hamster in addition to newborn mice during the initial isolation procedures. This illustrates the usefulness of the hamster as an additional experimental animal system for arenavirus study. Among the remaining South American arenaviruses, Pichinde is perhaps most significant for

the contribution to biochemical and biophysical studies. Primarily due to the efforts of Dr. W.E. Rawls and colleagues, more information became known regarding the chemical properties of this virus compared to other members of the group, although this lead has rapidly narrowed as modern techniques are applied to the study of all arenaviruses.

Arenaviruses isolated from rodents native to the American continents are often referred to as the 'New World' arenaviruses, distinguishing them from subsequent arenavirus isolations from the Old World. The most important of the latter is the agent of Lassa fever, a disease first described in 1969 in a missionary nurse stationed in the township of the same name situated in north-eastern Nigeria. The subsequent rate of transmission among doctors and nurses, a high mortality rate in early outbreaks, and a fear of rapid spread among individuals returning by air to the northern hemisphere from endemic areas, have resulted in a high degree of public emotion and awareness of this virus in both Europe and the U.S.A. This concern is exemplified by the publication of J.G. Fuller's book '*Fever*' (Fuller, 1974) for general reading. Hospitalization of the first recorded case resulted in two secondary infections among contacts: of these, one survived ('LP') following evacuation to the U.S.A. and provided the prototype Lassa virus strain and subsequently a source of immune plasma. In January 1970, a further outbreak occurred centred around the hospital at Jos, Nigeria. Among the 13 deaths was Dr. J.M. Troub, a physician who contributed an early description of the infection to the northern hemisphere from endemic areas, have resulted in a high breaks, attention has widened to other West African states. In 1972, an outbreak occurred in Liberia and, as had taken place in Nigeria, the cases were seemingly connected to a hospital. Only the index case had acquired the disease from elsewhere. Studies in neighbouring Sierra Leone have also shown that febrile illness among native populations can be ascribed to Lassa virus. It is now known that Lassa infection is more widely spread than at first thought and that in numerous cases of hospital-centred outbreaks the index case survives (Monath, 1975). Among these are descriptions highlighted by Dr. J. Casals which report on disease found among rodent catchers in areas bordered by present-day Upper Volta and the Central African Republic. That other viruses related to Lassa exist in the same natural host or close relatives has been shown by the finding of cross-reacting arenavirus isolates from Mozambique, Zimbabwe and the Central African Republic (Table 1.1). These new isolates apparently do not cause significant human disease in their natural setting, although such epidemiological studies are inevitably limited at the present time. All the arenavirus agents from the African continent share a much closer

serological relationship with LCM virus as compared to the Tacaribe complex arenaviruses. For this reason, LCM together with Lassa and other African arenavirus isolates are frequently referred to as 'Old World' arenaviruses.

1.2. Classification

The family arenaviridae was approved and given official status by the International Committee on Taxonomy of Viruses in 1970 and the morphological, physicochemical and serological criteria considered for the differentiation of these viruses from other groupings were summarized by Pfau et al. (1974). A further update on the family and its taxonomical relationship with other viruses is to be found in Matthews (1982) with a further update pending (W.E. Rawls, personal communication). The various strains and isolates of LCM virus are classified as a genus with the vernacular name. At the time of compilation, the relationship between LCM virus and other members of the family has become clearer, in particular the close serological relationship that may exist between this virus and Lassa virus from West Africa (see Chapter 3). The development of monoclonal antibodies is beginning to resolve more clearly these relationships and it may be expected that the full nucleotide sequence of at least LCM, Lassa and Pichinde virus genomic RNA will shortly become available (see Chapters 7 and 8) providing further information for classification and subdivision of the arenavirus family.

1.3. Nomenclature

With the exception of LCM (lymphocytic choriomeningitis) virus, almost all the arenaviruses so far characterized are referred to by names that reflect the geographical area from which they were isolated. The exception, perhaps is virus BeAn 293022 which is referred to in the early literature from the Belem Laboratory only by its assigned laboratory number but is now commonly referred to as Flexal virus (Matthews, 1982). A full listing of the arenavirus family, together with the origin of each name, is presented in Table 1.1. Various strain identifications are also commonly used, in particular those arenaviruses isolated from man and LCM virus, and some of these are listed in Table 1.2. These tabulations are not intended to provide a comprehensive listing, rather a reference point for the reader. Fewer multiple isolations have been made of

TABLE 1.3.
Data on commonly studied Tacaribe complex viruses

Virus	Strain	Place	Date	Plaque size
Tacaribe	11537	Trinidad	March 1956	Small
+ 19 other strains from bats and mosquitoes				
Pichinde	3739 'prototype'	Pichinde valley, Columbia	Aug. 1965	Medium
	4763	Munchique mountains, Columbia	Sept. 1970	Medium
+ seven additional strains isolated between Sept. 1970 and April 1971				
Tamiami	W-10777	Florida, U.S.A.	Sept. 1975	Small
Amapari	BeAn 70563	Amapa, Brazil	July 1964	Small
+ 13 other strains isolated from *Neacomys* and *Oryzomys*				

non-pathogenic viruses infecting New World rodents only. The exception to this is the number of Pichinde isolates obtained by Dr. Sanmartin and his colleagues whilst working in Cali, Columbia. These and isolates of other non-pathogenic arenaviruses are given in Table 1.3.

CHAPTER 2

Natural history

The natural history of arenaviruses is dominated by the characteristics of rodent infection, and the unique restriction to a limited number of rodent species. A summary of rodent species known to be associated with arenaviruses is given in Table 2.1. Although there are some 1700 species of rodents world-wide grouped into 34 families, arenaviruses are associated with only two families – the Muridae and the Cricetidae – although it has been pointed out that these constitute the bulk of rodent life (Arata and Gratz, 1975). The cricetid rodents of importance in the natural history of arenaviruses are found in the South American subcontinent and represent rather recent additions to the rodent fauna of that region, having crossed the Panamanian isthmus sometime in the Miocene or Pliocene era (Hershkovitz, 1966). The original rodent fauna of South America was largely composed of members of the New World hystricomorphs, whose present day members include the guinea pigs. Of particular note here is that although laboratory-bred guinea pigs are susceptible to Junin and Machupo infections, there is no evidence that this species plays any significant role in the natural history of either virus. Both Muridae and Cricetidae families are divergent from the ancestral Myomorpha line of rodents, which also include rats, hamsters and jerboas. A major part of their ecological success is related to the evolution of a powerful jaw musculature and dentition suitable for a wide variety of ecological niches. The cotton rat (*Sigmodon hispidus*) of Florida, the natural reservoir of Tamiami virus, has been shown to be fundamentally a South American cricetid rodent which has reinvaded back into North America (Ray, 1957, quoted in Arata and Gratz, 1975).

2.1. Arenaviruses pathogenic for man

Both Machupo and Junin may be transmitted to man from rodents with very different ecological patterns. Machupo is restricted to *Calomys callosus*, a mouse which favours the upper Savannah regions in eastern Bolivia and neighbouring South American countries (Hershkovitz, 1959). In the absence of other peridomestic rodents it inhabits gardens and houses with frequent opportunity for human contact.

Machupo virus has only been recovered from *Calomys* in the Beni region of Bolivia (Webb et al., 1967) suggesting a further geographical restriction in the parasite-natural host relationship, a feature in common

TABLE 2.1.
Species of mammals known to be associated with arenaviruses (data collated from Arata and Gratz, 1975; Johnson, 1981; Gonzalez et al., 1983)

Virus	Species	Location	Habitat
Old World arenaviruses			
LCM[a]	*Mus musculus*	Europe and the Americas	Peridomestic
Lassa[a]	*Mastomys natalensis*	West Africa	Savannah
Mopeia	*Mastomys natalensis*	Southern Africa	Savannah
Mobala	*Praomys* spp.	Central African Republic	Savannah
New World arenaviruses			
Junin[a]	*Calomys musculinus* *C. laucha* *Akodon azerae*	Argentina	Grasslands
Machupo[a]	*Calomys callosus*	Bolivia	Grasslands, peridomestic
Tacaribe	*Artibeus literatus* *A. jamaicensis*	Trinidad	Forest, periurban
Amapari	*Oryzomys goeldii* *Neacomys guianae*	Amapa, Brazil	Tropical forest
Flexal	*Oryzomys* spp.	Para, Brazil	Tropical forest
Pichinde	*Oryzomys albigularis* *Thomasomys fuscatus*	Columbia	Tropical forests, river valleys
Latino	*Calomys callosus*	Bolivia, Brazil	Grasslands, peridomestic
Parana	*Oryzomys buccinatus*	Paraguay	Tropical Forest, savannah
Tamiami	*Sigmodon hispidus*	Florida, U.S.A.	Marshlands

[a] Human pathogens.

with Junin virus in Argentina. The endemic area of Machupo virus is predominantly open grassland in an area where the annual rainfall may be as much as 6 feet. This savannah-like habitat is frequently flooded to a depth of 4 or 5 inches from January to May of each year, with only high ground remaining dry to form islands or 'alturas' (Chinel, 1978). *C. callosus* is essentially a pastoral species favouring scrub forest when the canopy is sufficiently open to allow a grass undergrowth. This rodent also frequents the small townships and agricultural areas on the periphery of the altura regions, where the forest canopy is often restricted by frequent burning. Interestingly, several species of *Oryzomys* are also found in this locality, a rodent genus from which Pichinde virus was isolated in Columbia (Trapido and Sanmartin, 1963). However, the *Oryzomys* species favour the brush forest which *C. callosus* does not penetrate.

Initial epidemiological investigations of Bolivian haemorrhagic fever showed a preponderance of rodents and bats in the homes of infected individuals. However, among the first six animals collected, all *C. callosus* rodents, two yielded positive isolations of virus (Johnson et al., 1966).

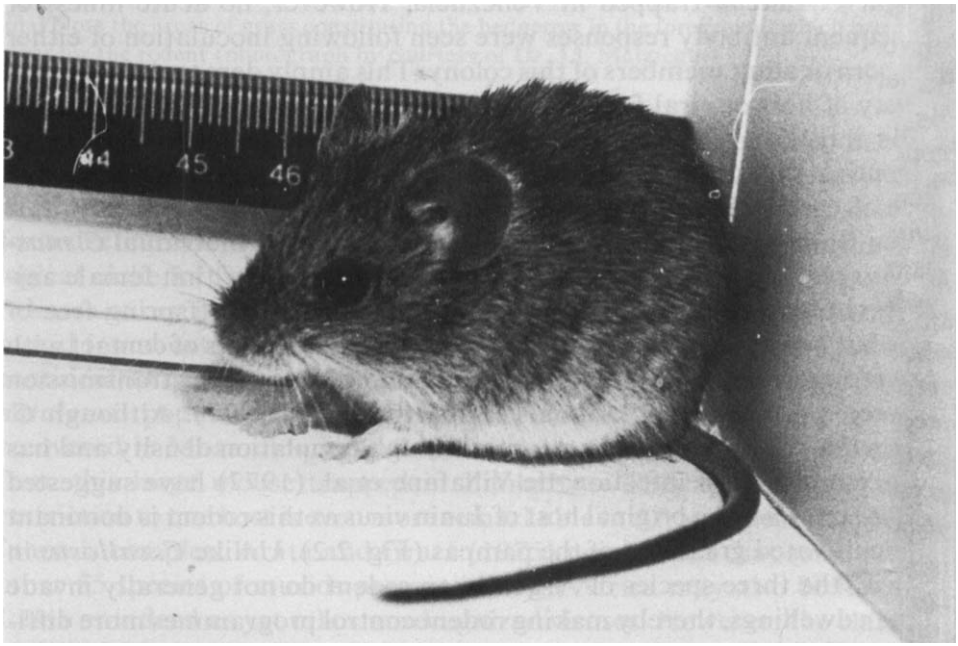

Fig. 2.1. The natural rodent host of Junin virus, *Calomys musculus*, endemic in the Pampas region of Argentina (photograph by courtesy of Dr. M.C. Weissenbacher).

There was no evidence of infection in bats, and indeed extensive surveys of rodent ectoparasites also proved negative (Kuns, 1965). The clear reduction in the numbers of new cases following the introduction of a rodent eradication programme (see Chapter 4) strongly indicated spread from infected rodents via urine contamination of soil and food. This mode of transmissions was also suggested by the recovery of virus from the urine of naturally infected animals (Johnson et al., 1965b).

The rapid decline in the local cat population with the advent of malaria control programmes over the entire province in 1959 may have contributed to a sharp rise in the local rodent population (Johnson et al., 1967). In 1963, only 12 cats could be found in the township of San Joaquin, a major focus of infection (Mackenzie et al., 1967). The absence of its natural predator as a result of DDT poisoning may therefore have directly contributed to the emergence of the zoonosis.

Junin virus has been repeatedly isolated from three cricetid rodents in the humid pampas of Argentina to the west and south of the river Plate delta. These are *Calomys musculinus* (Fig. 2.1) *Calomys laucha* and *Akondon azerae* (Sabattini et al., 1977). Interestingly, Webb et al. (1973) were able to infect animals up to 3 days of age bred from wild-caught *C. laucha* trapped in Venezuela. However, no acute illness or subsequent antibody responses were seen following inoculation of either newborn or adult members of this colony. This amply demonstrates that a variety of host or viral factors may play a role in establishing a chronic Junin infection. The population density of all three rodent species in Argentina fluctuates according to season, reaching a peak in May and June of each year to coincide with the harvesting of grass crops in this region. Junin virus has been successfully isolated from individual *C. musculinus* on repeated recapture. Laboratory studies show that female animals, viraemic at the end of pregnancy, give birth to offspring free of virus but newborns become infected after 3 or more weeks of contact with infected mothers. Evidence is also available of horizontal transmission between young or adult animals (Sabattini et al., 1977). Although *C. musculinus* is present in the pampas at high population density and has the greatest rate of infection, de Villafane et al. (1977) have suggested that *A. azerae* is the original host of Junin virus as this rodent is dominant in uncultivated grassland of the pampas (Fig. 2.2). Unlike *C. callosus* in Bolivia, the three species of Argentinian rodent do not generally invade human dwellings, thereby making rodent control programmes more difficult to effect. Human contact is therefore predominant among agricultural workers who tend and harvest the annual maize crop. Rodent numbers are more appropriately controlled by restricting weed growth among

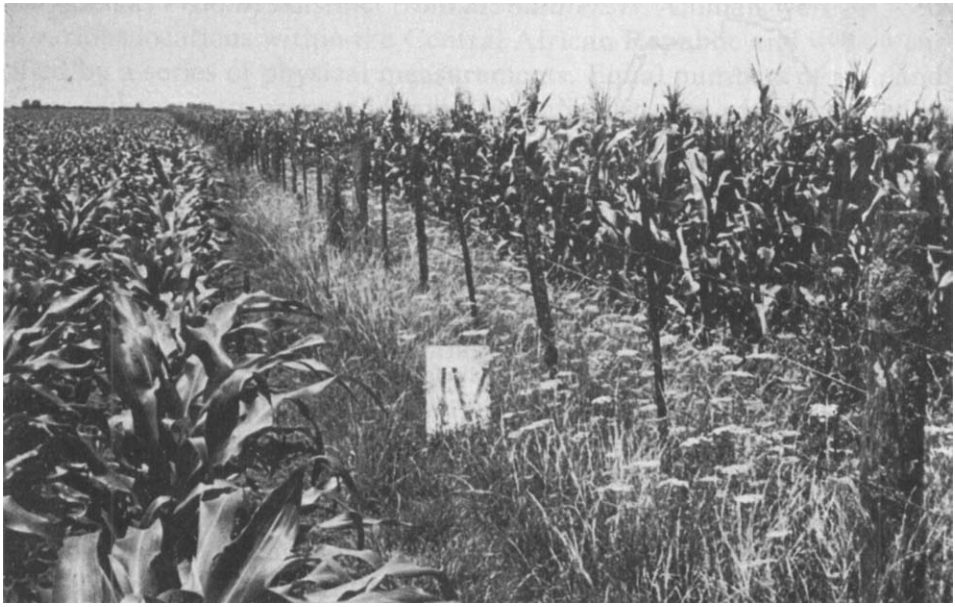

Fig. 2.2. Natural habitat of *Calomys musculus* in the maize-growing Pampas of Argentina. Note the areas of grass constituting the hedgerow in the foreground which provides cover for the rodent (photograph by courtesy of Dr. J.I. Maiztegui).

maturing maize plants and also attempting to restrict the rodent habitat on the periphery of cultivated areas. Changing agricultural practice, in particular the replacement of maize by soybean crops may be responsible for a decline and/or spread of Junin infection in recent years as the ecological niche for field rodents undergoes change.

Lassa virus is found only associated with *Mastomys natalensis*, a murid found extensively throughout the savannah region of the African continent (Fig. 2.3). Although Lassa virus has only been found in *M. natalensis* populations of West Africa, a related arenavirus originally recovered in Mozambique from the same species has also been isolated from this large mouse trapped in Zimbabwe. This apparent exquisite restriction to one host is even more remarkable in Africa where the rodent fauna is complex. Arata and Gratz (1975) pointed out that some of the other 55 species of murids present in West Africa may also be hosts of Lassa or indeed as yet other unrecognized viruses. However, the classification of *M. natalensis* is somewhat complex and there is some disagreement between zoologists as to the exact relationship between various genera within the family Muridae. For example, *Mastomys* is frequently not

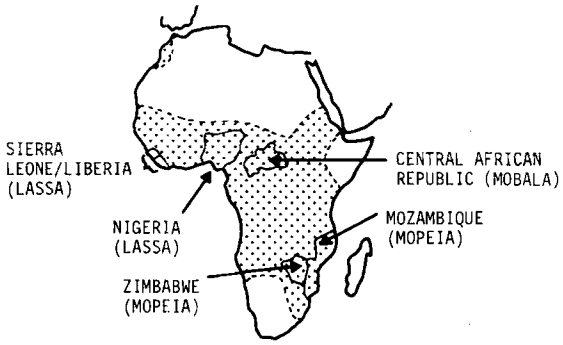

Fig. 2.3. Geographical locations of Lassa and related viruses from Africa. ⊡, Distribution of the major rodent host *Mastomys natalensis* in this continent (from Howard and Young, 1984).

regarded as a genus sufficiently distinct from other murids, and is often grouped together with other species into a single genus *Praomys* on the basis of similar dentition and jaw-bone structure. This has often resulted in the natural rodent carrier of Lassa to be referred to as *Praomys (Mastomys) natalensis* (Davies, 1965). An alternative view is that *Mastomys* is sufficiently distinct by karylogy to warrant the status of an independent genus (Rosevear, 1969). Further differentiation can then be made on chromosome number, with *M. natalensis* possessing $2n = 32$ chromosomes and the related species *M. coucha* being distinguished by $2n = 36$. Although these distinct *Mastomys* genotypes may be found in discrete geographical areas, both may co-exist in the same locality (Tranier, 1974; Green et al., 1978). In the study of Johnson et al. (1981) final identification of trapped rodents tentatively identified as belonging to the *Mastomys* complex were differentiated by electrophoretic analysis of liver haemoglobulins which may be used to confirm a positive identification of *M. natalensis*. It is unclear if there is a preference for Lassa and other African arenaviruses for either species, although preliminary work suggests that both species of *Mastomys* are associated with Lassa and there is no preference of infection for either genotype (Johnson et al., 1981).

The system and nature of rodent classification is varied and in urgent need of review, and *M. natalensis* should therefore be regarded as representing a complex of species in general rather than one distinct genus. The problem of rodent identification together with the search for Lassa and related agents in the rodent fauna is illustrated by the report of Gonzalez et al. (1983) who isolated several arenavirus strains from rodents

classified as *Praomys* distinct from *M. natalensis*. Animals were trapped in various locations within the Central African Republic and were identified by a series of physical measurements. Equal numbers of sera and tissue extracts were prepared for virus isolation from animals classified as *Praomys* or *Mastomys*, but no positive isolates were recorded from the latter. Almost all of the positive isolations were obtained in areas where the population of *Praomys* was highest, notably in border zones between forest and savannah. Also antibodies cross-reactive with Lassa or Mopeia (Mozambique) arenaviruses could not be detected in *Mastomys* rodents. A total of eight isolates were identified, all from *Praomys* tissues. Unlike antigens of Mopeia virus, none of these isolates from the Central African Republic reacted with monoclonal antibodies which identified Lassa virus (Chapter 3, Section 3.2). Although this demonstrates that the Central African viruses may indeed represent distinct new members of the family arenaviridae, it is clear that the natural host is also distinct. Further rodent trapping studies have been carried out elsewhere in the African continent in order to determine the extent of distribution of Mopeia and related agents outside of Mozambique. For example, Johnson et al. (1981) have successfully isolated six strains of Mopeia virus from around two townships in Zimbabwe. All were isolated from *Mastomys*, confirming that these viruses are most likely primary infections of this rodent species. Antibodies to the Mopeia virus were found in approximately 10% of all rodent sera examined, including some from the related rodent *Aerhomys chrysophilus*. Interestingly, no antibody was present as revealed by immunofluorescence in those animals from which virus was recovered.

M. natalensis has received attention because of its intermediate ecological niche between the African Savannah and urban areas. Often occupying burrows of other rodents, it has been extensively implicated in the transmission of bubonic plague (Isaacson, 1975). The animal is essentially nocturnal, feeding on grass seeds and readily invades sheds and granaries used to store corn and millet for human consumption. The exceptionally high litter size during the rainy season makes this rodent one of the most successful in Africa. Although timid towards many animals, it is aggressive in the laboratory and will readily bite. The clean behaviour pattern of this animal is reflected by a tendency to leave its burrow to urinate and defecate, which may have implications in understanding transmission of Lassa fever virus to man (Isaacson, 1975). Keenlyside et al. (1983) have looked in detail at the infection rates among *Mastomys* rodents trapped in and around households where members are known to have clinical Lassa infection.

Nearly 80% of all rodents trapped during the course of this study were identified as *Mastomys* species. In such houses, approximately 40% of animals appeared to be persistently infected with the virus with a further 172 positive for viral antibody. This contrasted with 4% with viraemia and 19% positive for antibody in animals caught in houses with no evidence of acute human infection.

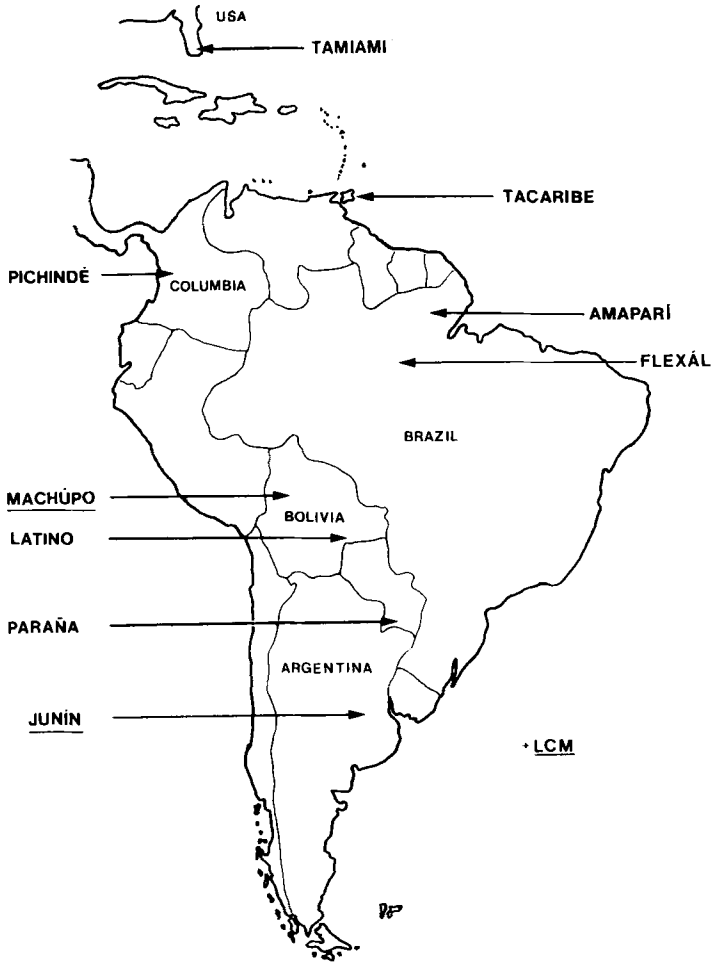

Fig. 2.4. Geographical isolations of arenavirus isolates in the New World. All viruses with the exception of LCM virus are serologically defined as being members of the Tacaribe complex (from Howard and Simpson, 1980).

LCM is predominantly associated with the common house mouse, *Mus musculus*, a rodent which originated in the Old World but is now closely associated with man on every continent. There have been reports of isolates from two species of the closely related *Apodemus* family (Lehmann-Grube, 1971) but such reports are rare. Much more important from the public health point of view has been the finding of LCM in laboratory-bred Syrian hamsters (*Mesocricetus quaratus*) from which the virus may be readily transmitted to man with serious consequences (Lewis et al., 1965; Ackermann et al., 1972; Baum et al., 1966; Hotchin et al., 1974; Biggar et al., 1975; Deibel et al., 1975; Hinman et al., 1975). However, LCM virus is not known to be associated with hamsters in the wild, although the infection may be readily transmitted from mice to hamsters (Skinner et al., 1976). There is also some evidence that infection may occur in humans after contact with apparently healthy hamsters (Blechschmidt et al., 1977). There is extensive natural infection of *Mus musculus* at least in its European habitat. Ackermann et al. (1964) reported that 4% in nearly 1800 animals trapped in Germany had signs of LCM infection. One problem in assessing the degree of zoonotic human LCM infection may relate to inadequate clinical awareness and/or the general 'influenza-like' nature of the symptoms (Ackermann, 1973). Human LCM infection has also been diagnosed within the endemic area of Argentine haemorrhagic fever and Maiztegui et al. (1975) have indicated the necessity of careful dissection of serological responses in epidemiological studies where LCM coexists in the local rodent population with a second arenavirus pathogenic for man.

2.2. Arenaviruses non-pathogenic for man

The majority of the arenaviruses so far identified have been isolated in the New World (Fig. 2.4). Amapari virus was isolated from tissues of an adult *Neacomys guianae* trapped in the proximity of a mining community north of the Amazonian delta (Pinheiro et al., 1966). Several additional isolates from *Oryzomys* spp. were also recorded, all animals being trapped in an area of secondary forest growth between a local hospital and a river which had become part flooded during the rainy season. There appears to be some minor differences between isolates from these two species. Only about 30% of suckling mice die after inoculation with isolates from *Neacomys* whereas the infection with isolates from *Oryzomys* were invariably fatal (Pinheiro et al., 1977). The carriage rate in both species appeared to be about 3–12%. However, longitudinal studies of

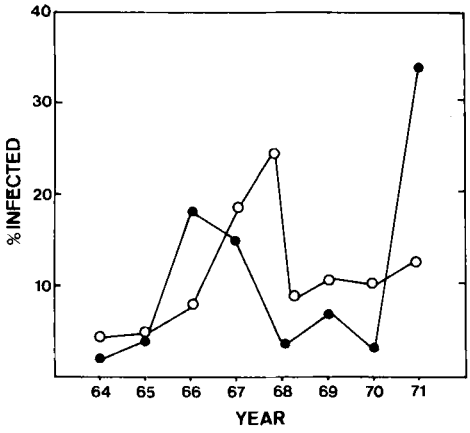

Fig. 2.5. Annual variation in the rates of isolation of Amapari virus from rodents trapped in the Amazon region of Brazil (redrawn from Pinheiro et al., 1977). Isolations are shown from *Neacomys* (○–○) and *Oryzomys* (●–●) species.

naturally infected animals showed that there was considerable oscillation in the level of circulating virus, with infectivity occasionally becoming undetectable in either blood or urine. Interestingly, the annual infection rate in both *Oryzomys* and *Neacomys* spp. appear to cycle (Fig. 2.5; Pinheiro et al., 1977).

A second arenavirus isolate has been described from Brazil, isolated from *Oryzomys* inhabiting a tropical rain forest close to the Trans Amazon highway (Pinheiro et al., 1977). Originally known by its laboratory code (BeAn 293022), this agent is distinct from Amapari and is known as Flexal after a local tributary. This may also represent a single host-parasite relationship, there being no evidence of this virus in other rodent species trapped in the same area. Similarly, Pichinde virus was isolated from *Oryzomys albigularis*, a rodent which favours the broader regions of the riverine valleys draining the central uplands of Columbia (Trapido and Sanmartin, 1971).

As Pichinde virus has been extensively characterized at the biochemical level, it is worth considering the details of the original isolation. The name refers to the mountain valley where the isolates were made, and this is described by Trapido and Sanmartin as an upland area covered extensively by secondary forest resulting from the production of regenerating small plants. Much of this area is referred to as fog forest and is a major catchment area for the supply of water to the city of Cali and surrounding areas. The authors found that Pichinde virus could be isolated in the

laboratory equally effectively in infant mice, infant hamsters and Vero cell cultures, and positive isolations were made from many organs of trapped animals including the brain, liver, spleen and kidney. The host range was found to be very specific: of 55 isolations only one came from a species other than *O. albigularis* although a total of 23 different species of small mammals were caught. The single isolation from *Thomasomys fuscatus* represents isolation from a rodent closely related to *Oryzomys*, although it is noticeable that the virus was not found in over 1000 specimens of different species of the *Oryzomys* family. In comparison, the overall isolation rate from *O. albigularis* was approximately 20% in 271 animals. Field studies showed that the virus was distributed equally among both male and female animals and was found in the rodent population all year round with the possible exception of May, an observation entirely consistent with the now recognized capacity of arenaviruses to induce chronic infections in their natural hosts. Several isolations were made from pools of ectoparasites, notably *Gigantolealaps* species and *Ixodes tropicalis*. Despite the prolonged viraemia and shedding of the virus in the urine, there is no evidence of antibody to this virus among the local human population and no positive cases among residents of the Pichinde valley itself. However, the significance of this is somewhat limited given the relative insensitivity of the serological tests employed in these studies. Certainly there is evidence that Pichinde can cause asymptomatic human infection, albeit in a laboratory setting (Buchmeier et al., 1974).

Trapido and Sanmartin (1971) selected their designated isolate CoAm 3739 as the Pichinde virus prototype. Owing to its relatively good growth properties compared to other Pichinde isolates, the prototype has received most attention from the biochemists. In addition, a further isolate has also been examined on the *a priori* assumption that its properties may differ somewhat because of its isolation 100 km distant in the Munchique mountains (Vezza et al., 1980; Table 1.3, page 8). The authors suggested that geographical separation of these isolates away from the original area of study may indicate the unique relationship between Pichinde virus and its host with possible stability in evolutionary terms.

As the *Oryzomys* species is found as far as Costa Rica to the north and as far south as Bolivia, the extent of Pichinde virus infection among the rodent population may be higher than suggested by these studies. Certainly other arenavirus isolates have been made from a different species of the same family caught elsewhere. Webb et al. (1970) found new arenavirus strains in *Oryzomys buccinatus* during a study designed to define the geographical boundaries of Machupo virus in *C. callosus* populations. The new isolates were collectively referred to as strains of Parana

virus, so-called after the Parana river in the region of isolation which forms the boundary between Paraguay, Brazil and Argentina. Unlike the high altitude of the area studied by Trapido and Sanmartin, however, Webb et al. trapped in a region of mature secondary forest only 300 ft above sea level. A further isolation was made some distance away near the border with Argentina and Bolivia. The virus was found in *O. buccinatus*; five other cricetid rodent species proved negative. As with Pichinde virus in Columbia, no serological evidence of human infection was found in a limited study of selected human sera.

Apart from the introduction of LCM virus by man, Tamiami virus is the only arenavirus indigenous to the rodent fauna of North America so far isolated. The original prototype reported by Calisher et al. (1970) was one of nine isolates obtained from cotton rats (*Sigmodon hispidus*) caught near the Tamiami trail, a major highway crossing the Florida everglades.

Tamiami virus was also isolated no fewer than 26 times over the period 1963–1967 by the Florida State Board of Health from cotton rats caught in a newly cultivated area of wetlands in the Tampa Bay area (Jennings et al., 1970). The authors reported that isolation of virus in suckling mice was frequently enhanced by prior dilution of tissue extracts, suggesting a high proportion of non-infectious particles in the organs of the persistently infected cotton rat. Wild-caught rodents were found frequently to contain antibody to Tamiami virus, particularly among adult animals. Some rats secreted infectious virus in the urine despite the presence of serum antibody thus providing a sensitive method for detecting infected animals. However, these findings with Tamiami virus are not universal; Murphy et al. (1976) using animals trapped in Georgia found that newborn cotton rats never excreted virus in the urine although infectious virus was present in many tissues. Adult animals had little or no virus present either in blood or in organs. These discrepancies may in part be due to host factor variation within the same species of rodent. For example, Webb et al. (1973) found that *C. laucha* caught in Venezuela did not support either acute or chronic Junin infection, despite the fact that this same rodent species represents a major natural reservoir of Junin virus in Argentina.

The cotton rat is common throughout the low-lying marshlands of Florida and Louisiana, feeding chiefly on agricultural crops and favouring thick grassy areas and banks for making their burrows. They are semicolonial and tend to share common runways, thus coming into contact with virus shed in the urine and faeces of passing animals. Cotton rats are known to be very aggressive and cannibalism is frequent. Both patterns of

behaviour may aid dissemination of the virus and the high isolation rate in young animals suggests that this may occur shortly after birth (Jennings et al., 1970). Despite the close proximity of cotton rats to man and the potential transmission onto contaminated crops, there is no evidence to implicate Tamiami virus as a human pathogen. This arenavirus-host relationship thus provides a further example of exquisite restriction of an arenavirus in causing an infection in one specific animal species.

It has been suggested by Murphy et al. (1976) that Tamiami virus infection of the cotton rat may represent an intermediate step toward immunotolerance and virus persistence, and that vertical transmission may require a certain degree of acceptance of viral antigen load. Both Machupo and Latino viruses similarly represent single host-parasite relationships which have been studied closely by Webb et al. (1975) in order to analyse the role of immunotolerance in persistence of the virus in nature. Experimental infection of *C. callosus* older than 9 days with Machupo virus produces a 'split response' (Justines and Johnson, 1969; Johnson et al., 1973). Whereas approximately half respond by clearing the virus after infection and subsequently become immune, the remainder were characterized as having persistent viraemia with little or no specific antibody. Excretion of virus from animals that do not respond immunologically is the prime source of virus for human infection. Fertility among this group is considerably reduced, indicating that Machupo persistence is a significant regulatory factor in controlling rodent population size (Webb et al., 1975). The virus appears to be sexually transmitted from persistently infected males to normal females and immunotolerant pregnant females produce only 5% of the expected number of offspring, due to failure of fertilization and/or failure to implant in the uterus. Although persistently infected females could be successfully impregnated, the majority of embryos were dead by parturition and there was considerable evidence of virus in embryonic tissues and the surrounding placenta. However, the embryos are protected from infection once placental enclosure has occurred. The prolonged excretion of virus from persistently infected animals maintains the presence of virus in the animal community although susceptible animals of the same species are required for its maintenance in the population.

Infection of suckling *Calomys* animals resulted in viraemia and little detectable humoral response; this is in contrast to Latino infection of similar animals which produces a chronic, but immunologically nontolerant infection. This virus was isolated also from *C. callosus* caught in three separate sites 200 miles to the south and east of the region endemic for Machupo virus (Webb et al., 1973; see Fig. 4.5, page 65). Prior infec-

tion with Latino virus does not alter the outcome of subsequent infection with Machupo virus and the capacity to produce either tolerant or non-tolerant chronic infection *Calomys* appears to be virus-specific. Webb et al. (1973) reported that *C. callosus* from regions far removed from the endemic region of Bolivian haemorrhagic fever were equally susceptible to Machupo virus, and made the observation that there is potential for spread of this virus to other, more fertile agricultural regions in Bolivia as communications improve. The already existent Latino virus endemic in the more southerly areas would not prevent the establishment of chronic Machupo infection in the *C. callosus* population of that region.

Webb et al. (1975) drew two very pertinent conclusions relevant to the understanding of arenavirus natural history; firstly, the quite different outcome resulting from the infection of suckling laboratory mice with LCM virus may be due to host factors inadvertently selected by breeding and that other features common between the two systems may be more relevant, such as anaemia, reduced lifespan and reduced fertility of animals infected early in life. Secondly, the study of Latino virus, apparently non-pathogenic for man but sharing the same vertebrate host is unique among arenaviruses in not apparently causing infection in the laboratory mouse. Indeed, this agent would not have been detected without the use of suckling hamsters, and this raises the possibility that many other arenaviruses await to be characterized.

The anomaly among the arenaviruses is the natural history of Tacaribe virus, this being the only member of the family to be isolated from a non-rodent source. The virus was isolated by the Trinidad Regional Virus Laboratory from two species of bat (*Artibeus lituratus* and *A. jamaicensis*) during a continuous vigilance for rabies virus among the urban bat populations (Downs et al., 1963). The first strain, designated TRVL 11573, was obtained from the brain of an animal caught in the Port of Spain, and subsequent isolations were all made from either brain or salivary gland homogenates, although one strain, number 13537, was made from a pool of mosquitoes collected in the forested region to the east of Trinidad. Yet in this study, no evidence was found of Tacaribe virus infection in any of 2000 species of indigenous mammals, mainly rodents, examined during a period of 7 years. As these species of bats are not carnivores, the possibility of the virus having been acquired by the hunting of small mammals is unlikely, although the possibility that virus spread resulted from contact with rodents sharing a similar nomadic habit cannot be excluded. Alternatively, this virus may represent a true pathogen of a small non-rodent animal.

CHAPTER 3

Laboratory diagnosis and serological properties

Early recognition of arenavirus infections is essential, particularly from the public health point of view and also to allow possible use of immune plasma and chemotherapy. In addition, sporadic cases among travellers returning to the northern hemisphere from endemic regions make rapid diagnosis and subsequent exclusion of arenavirus involvement an urgent requirement, particularly if febrile illness due to parasitic or another microbiological pathogen is suspected.

In the event of tests for arenavirus and other more common infections giving negative results, caution is still required particularly with specimens from severely ill patients with a clinical history suggestive of recent visits to tropical areas. Several highly dangerous pathogens, e.g. Ebola virus, have emerged with little warning and there is every indication to suggest that many previously recognized viruses may emerge in new localities with enhanced virulence, e.g. recent outbreaks of Rift Valley fever in the Sudan and Egypt.

3.1. Safety considerations

It is to be emphasised that clinical material from individuals suspected of being infected with arenaviruses must only be handled in an appropriate containment facility recognized by the national public health authorities as pertaining to the required degree of microbiological security. For

TABLE 3.1.
Centres with maximum containment facilities for handling dangerous pathogens

Location	Country
Centre for Applied Microbiology and Research, Porton Down	U.K.
Centers for Disease Control, Atlanta, Georgia	U.S.A.
U.S. Army Research Institute for Infectious Diseases, Fort Detrick, Maryland	U.S.A.
Institute of Tropical Medicine, Antwerp	Belgium
National Institute for Virology, Johannesburg	South Africa
National Institutes of Health, Tokyo	Japan
Ontario Public Health Laboratories, Toronto	Canada
Institute of Poliomyelitis and Encephalitis, Moscow	U.S.S.R.

example, the Centers for Disease Control, Atlanta and the Center for Applied Microbiology and Research, Porton Down, are recognized category A (P4) facilities for the United States and the United Kingdom public health services respectively.

These and other centres are listed in Table 3.1. This list is not comprehensive, but is a guide to those laboratories with diagnostic and research interests in arenaviruses and other agents causing haemorrhagic fever. Smaller containment laboratories exist, e.g. in various centres of the U.K. Public Health Laboratory Service, but facilities are more limited. In addition, maximum security laboratories exist in many countries for the handling of economically important agricultural pathogens. These offer an additional resource of containment facilities for handling human pathogens in a national emergency.

Diagnosis of human arenavirus infections and other rare but highly contagious pathogens is coordinated by the World Health Organisation, Geneva, Switzerland, where appropriate advice may be sought regarding the most suitable laboratory for receiving specimens according to geographical locality. Effective use of facilities requires prior contact with the receiving laboratory in order that the appropriate laboratory facilities may be made available to receive material and that guidance may be offered in regard to the packaging and despatch of biological fluids sus-

TABLE 3.2.
Summary of laboratory-acquired arenavirus infections up to 1979

Virus	Total infections	No. inapparent infections	No. deaths
Lassa	2	0	1
Junin	21	5	1
Machupo	1	0	1
LCM	15	2	0
Pichinde	17	16	0
Tacaribe	2	1	0

pected of containing the relevant infectious agent. One particular problem is the use of commercial air carriers for shipment between countries: the International Air Transport Association (IATA) requires strict adherence to published guidelines for packaging and shipment of potentially hazardous biological material, including viruses and other restricted articles.

Although this monograph primarily aims to describe arenaviruses and their infections it is appropriate to mention briefly the problems of safety and containment allied to the handling of certain arenaviruses pathogenic for man. In particular, transmission of arenaviruses may occur via the generation of aerosols, and the avoidance of contamination of mucosal surfaces, skin and clothing of workers handling potentially positive clinical specimens is one of the major principles in biological containment. Table 3.2. summarizes the number of infections thought to have occurred up to 1979 as a result of laboratory handling of arenaviruses. Deaths have been associated with the handling of material from human cases, and at least eight cases overall have been attributed to aerosol contamination. Modern equipment, such as centrifuges specially designed for cell washing and routine haematology, all generate aerosols which are potentially hazardous when used in an open laboratory for the preparation of specimens thought to contain pathogens (Harper, 1981). The approximate dose required to infect man with most of the pathogenic arenaviruses by aerosol or any other route is not known; however, the very high levels of viraemia seen in patients infected with Lassa virus compared to other human arenavirus infections make fluids from such patients potentially a potent source of airborne contamination.

The design of a suitable laboratory which offers the required degree of containment has been recently described in detail by Wright et al. (1982). Of particular concern is the exclusive use of totally enclosed safe-

ty cabinets within such facilities. Indeed the so-called Class III cabinets are also to be recommended for the handling of certain other arenaviruses if large quantities of concentrated virus are being handled or there is a need to use procedures which may result in the generation of aerosols, e.g. resuspension of viral pellets, sonication of infected cells. In such closed systems, any virions released on airborne droplets will be retained on high efficiency particulate air (HEPA) filters as the air is exhausted from the cabinet to the outside environment. Adequate treatment of sewage together with an air-lock to the entrance of such facilities are additional features of a high security laboratory and workers are required to change clothing prior to entry and to take a shower on exit. An alternative design for such a laboratory consists of workers wearing so-called 'space-suits', each with an individual air supply from a common line.

Patient isolation, together with the use of strict barrier nursing techniques is an essential first precaution in the care of infectious individuals, particularly those suspected of Lassa infection. Documentation is available giving advice on the management of suspected or confirmed cases of Lassa fever, e.g. Morbidity and Mortality Report (1980) issued by the U.S. Department of Health, Education and Welfare. Transportation of suspected cases from endemic areas to Northern Europe and the U.S.A. has been successfully achieved using completely enclosed isolators manufactured from transparent flexible film employing principles originally developed for the raising of pathogen-free livestock (Trexler et al., 1977). Such units are now being used increasingly within containment laboratories as an alternative to rigid cabinets (van der Groen, 1980). Less rigorous facilities are recommended for arenaviruses considered as being non-pathogenic for man, and the appropriate containment levels are summarized in Table 3.3. Although many of the arenaviruses non-pathogenic for man may be regarded as relatively safe to handle in a virus laboratory with minimal safety equipment, it should be stressed that many of these viruses may be potentially hazardous once grown and concentrated in large amounts, e.g. for biochemical studies. In these circumstances a higher level of containment is advisable and the use of safety cabinets recommended whenever possible to reduce the chance of aerosol contamination. There has been one incident cited where Tacaribe virus infected a laboratory worker handling a roller culture flask containing the virus, probably by aerosol generated on opening the bottle. Although many other members of the Tacaribe complex are regarded as 'level 2' pathogens, it should be noted that only LCM, Pichinde, Tacaribe and to

TABLE 3.3.
Recommended containment levels for the safe handling of arenaviruses

Virus	Containment level	Justification
Lassa Junin Machupo	Rigorous security; filtered air and treated effluent Double barrier between samples and workers. Shower at exit; complete change of clothing. Double-ended autoclaves (level 4)	Usually severe, frequently fatal human infections; aerosol transmission documented
LCM[a]	Restricted access to segregated area under negative air pressure; high efficiency filtration; use of safety cabinets. Dedicated laboratory clothing (level 3)	Potentially severe or life-threatening human infections
LCM[a] Pichinde[b] Tacaribe[b] Tamiami Amapari Latino Parana Flexal	Controlled access; segregated work area with trained personnel; use of safety cabinets where possible (level2)	Naturally acquired infections uncommon. Risk of infection can reasonably be assessed[b]

[a] Considerable differences exist between different laboratory strains of LCM virus. Many investigators regards neurotropic strains as requiring the higher level of containment.
[b] Highly concentrated preparations for, e.g. biochemical studies handled at a higher containment level in many laboratories. Also manipulations likely to result in excessive aerosol generation, e.g. sonication.

some extent Tamiami, viruses have received any degree of attention. Problems may still arise once remaining members are more widely studied. For example, Latino virus was originally isolated from the same species of rodent which is the natural host of Machupo virus. Also, there is some evidence to implicate Flexal in a laboratory-acquired infection (Pinheiro, 1982).

3.2. Diagnosis of human arenavirus infections

3.2.1. Lymphocytic choriomeningitis

Virus may be successfully isolated from blood during clinical illness and

additionally from cerebrospinal fluid if neurological symptoms are present. Since 1960, LCM of man has only been reported in the U.S.A. among individuals exposed to hamsters persistently infected with LCM virus. Direct virus isolation may be attempted by inoculation of mouse L cells (Hotchin, 1971b) but the mouse remains the most sensitive indicator; convulsions and death may occur within 5 days of inoculation by the intracerebral route. However, there is considerable variation in response, some animals exhibiting a much slower disease progression and yet others showing no sign of clinical illness despite being persistently infected. Hotchin and Sikora (1975) stated that this may be overcome by challenging test animals with a preparation of *E.coli* endotoxin. Those animals infected with LCM virus become exquisitely sensitive to normal, nonlethal doses of toxin (Hotchin, 1962). The endotoxin is given intraperitoneally 7 days after intracerebral virus inoculation of young adult mice and observing for death 24 h later. Endotoxin may be used earlier, but a higher dose is required and there is some loss of sensitivity. These procedures have been of value in the diagnosis of human LCM (Hotchin et al., 1974).

Although laboratory out-bred guinea pigs are often recommended for the direct isolation of arenaviruses and other so-called exotic pathogens, the response to LCM virus is variable. Most strains and clinical isolates do not cause overt disease despite virus replication in various tissues. One exception is the WE-strain which causes death within 12 days of inoculation (Kirk et al., 1980).

In man, complement-fixing (CF) antibodies are seen from one week after onset of illness, reaching a maximum by 6–8 weeks in the majority of cases. CF antibody levels then decline rapidly to disappear within 6 months (Smadel and Wall, 1940). The relatively short time course of CF antibody presence as compared to neutralizing antibody is therefore of some diagnostic value, with a positive result indicating ongoing or recent LCM infection (Rasmussen, 1947), although in some cases CF antibody may persist. On occasions, CF antibody is not found despite positive virological confirmation of disease (Cohen et al., 1966; Rasmussen, 1947; Skinner et al., 1976), presumably as the result of test insensitivity and/or the simultaneous presence of antibodies specific for the antigen but which fail to fix complement (Schmidt and Harding, 1956).

Quantitation of antibody by the CF test may be readily accomplished by determining that dilution of serum which fixes all or a defined proportion of complement added to the test, providing the total concentration of antibodies is below the limiting concentration of antigen. The source of reference antiserum may provide some problems, as animal hyperim-

mune antisera frequently contain inhibitory or anti-complementary activity. However, mouse antisera particularly sera from persistently infected animals subjected to adoptive immunization (Volkert et al., 1964) are often suitable having very high titres of specific CF antibodies. The preparation of suitable CF antigen may be accomplished using either infected cells or extracts of tissues from infected animals.

A much more sensitive method for detecting LCM virus antibodies in human sera has been described by Blechschmidt et al. (1977). Using a hyperimmune rabbit antiserum as a source of antibody and a commercially available tissue extract prepared from infected guinea pigs, a radioimmunoassay was developed whereby specific antibodies in human sera were detected by competition with a limiting concentration of radiolabelled rabbit antibodies. The viral antigen was immobilized on a polystyrene solid phase previously coated with antibody. This method was found to be approximately 32 times more sensitive than CF methods for antibody detection, although the radioimmunoassay did not display the same degree of broad specificity as the CF test. Somewhat greater claims of increased sensitivity of radioimmunoassay (and enzyme-linked immunoassay) have recently been made by Ivanov et al. (1984) who found up to 200-fold and 2000-fold increase in sensitivity for the detection of antigen and antibody respectively. Van der Zeijst et al. (1983a) have described an experimental radioimmunoassay system that would also be easily transposed into a system suitable for epidemiological studies. The format consisted of a sandwich-type of immunoradiometric assay using an immune serum from hamsters and the antigen was added directly. Bound antigen was subsequently detected by addition of radiolabelled antiviral IgG. This assay could be readily modified for the detection of viral antibody in test specimens by competition for the labelled Ig and after appropriate standardization of the antigen source. In addition, the same degree of sensitivity may be achieved by adaptation to a technically similar enzyme-linked immunoassay (ELISA).

Early studies designed to detect and quantitate neutralizing antibodies to LCM virus showed that this reactivity is thermolabile. For example, Ackermann et al. (1962) found significant decreases in log neutralization indices of positive human sera after heating for 20 min at 56°C. Some loss was also seen on prolonged storage at 4°C: at this temperature the titre of CF antibodies remains unchanged (Scheid et al., 1959). An unexplained phenomenon was the finding by Ackermann et al. that thermal inactivation at elevated temperatures could be partially reversed by subsequent storage at room temperature or below. Longitudinal studies have shown that neutralizing antibodies reach a maximum by 3 months from onset of

illness and persist at a high level for many years (Scheid et al., 1960). In the absence of critical data outlining the development of IgM neutralizing antibodies, these studies showed the limitation of single samples for serodiagnosis using neutralizing antibody as the sole marker of infection. In addition, the studies of Scheid et al. (1960) showed that most human sera contain natural inhibitors of virus replication. When mixed with undiluted human serum, the titre of LCM virus was found to be reduced by as much as 2 logs. On exposure to virus, the concentration of these inhibitors remain at the same level whereas specific antibodies may eventually produce log neutralization indices as high as 5.

Many early results were obtained by injection of virus-serum mixtures into the footpads of susceptible mice, a technique found to be much more sensitive than direct intracerebral inoculation although results require 3 weeks to develop (reviewed by Lehmann-Grube, 1971). It was found that positive neutralization prevents the development of generalized infection with the result that immunity to intracerebral challenge does not develop although local footpad immunity may be demonstrated (Hotchin, 1962). The footpad inoculation technique has been replaced by a more rapid microneutralization procedure whereby results may be obtained within a working week (Hotchin and Kinch, 1975). Optimal performance of this technique requires mixing of virus with antibody dilutions directly in plastic microtitre plates, addition of agarose, followed by a further agarose overlay containing BHK-21 cells in suspension. Small plaques become apparent in control wells 4 days later after the addition of neutral red. This method is at least twice as sensitive as immunofluorescence for antibody detection; although immunofluorescence offers considerable advantages for rapid diagnosis of acute infection, the sharp decline of antibodies detected by immunofluorescence similar to CF antibodies makes the alternative use of methods for the detection of neutralizing antibody activity desirable, particularly for epidemiological surveys. It should be noted, however, that techniques which use the constant antibody-varying virus dilution approach to determine log neutralization indices are subject to several theoretical drawbacks (Fazekas de St Groth, 1962) and quantitation is best accomplished by methods using fixed virus-varying antibody concentrations. Following this approach, Lehmann-Grube (1971, p.138) described the quantitation of neutralizing antibodies by determining the reciprocal of that serum dilution which reduces virus infectivity by 2 logs.

3.2.2. Lassa fever

Diagnosis of Lassa fever needs to be rapid, as this is the most contagious

of human arenavirus infections, with frequent reports in early studies of secondary and tertiary cases. In addition to the need for isolation of suspected and confirmed cases, other pathogens may induce a disease pattern similar to Lassa fever which may complicate clinical diagnosis. These include yellow fever, typhoid and malaria (Monath, 1973).

Owing to the very high levels of virus present in the blood during the acute illness, isolation of virus is feasible by direct inoculation into Vero cell cultures. Infectious virus may be recovered from either serum or whole blood from the 3rd day, up to nearly 3 weeks from the onset of the illness (Buckley and Casals, 1970; Monath et al., 1974); virus may also be recovered from throat washings, and from urine up to 32 days from onset (Winn et al., 1975). Samples positive for Lassa virus induce a cytopathic effect within 96 h of inoculation and may be confirmed for specificity by prior incubation with specific antiserum (Buckley et al., 1970; Buckley and Casals, 1970; Henderson et al., 1972). The cytopathology is characterized by the rapid development of necrotic foci which eventually results in complete destruction of the cell monolayer within one week. Basophilic inclusion bodies may be readily seen within the cytoplasm during the advanced stages of infection, representing ribosomal material embedded in protein matrices. The infectivity may readily be quantitated in the same cell system, using an agar overlay to restrict virus spread. However, there are problems in the quantitation of neutralizing antibodies to this virus by the plaque number reduction method (see below, page 42).

Although serological differences may exist between isolates of different geographical origin, all so far isolated may be grown in Vero cells and is therefore the substrate of choice for direct virus isolation, although several studies have shown that other cell types may be equally susceptible. Van der Groen et al. (1978) found that Lassa virus replicated in a diverse variety of cell lines including viper, pig kidney and BHK-21 cells. A cytopathic effect was observed in Indian Muntjak cell cultures similar to that seen with the use of Vero cells. Lukashevich and colleagues (1983a) have reported that Lassa virus grows to equally high titres in Vero, mouse L and pig kidney cell lines as well as in human diploid and primary human embryo kidney cell cultures. Although BHK-21, HeLa and other continuous cell lines also supported virus growth, yields of infectious virus were found to be at least one log lower. The yields of virus were found to be somewhat lower in CV-1 cells as compared to Vero cell cultures, despite the similar origin of these cells from monkey kidneys and the formation of plaques in both cell systems. The virus failed to replicate at all in primary chick embryo fibroblasts, and there was no evidence of viral antigen accumulation to suggest an abortive infection.

Lassa virus antigen may be detected within the cytoplasm of infected cells by indirect immunofluorescence a few days after inoculation and prior to the appearance of cytopathic effect, thereby reducing the time required for making a positive diagnosis (van der Groen et al., 1978). Time is frequently of essence and Wulff and Lange (1975) reported that a positive diagnosis may be made by this technique within 72 h of receiving specimens in the laboratory. This test has also been used extensively to re-evaluate the prevalence of antibody in endemic areas, although fluorescent antibody titres decline rapidly with time (Frame, 1979; Frame et al., 1984). The 'half-life' has recently been estimated at 3 years (Jahrling et al., 1985).

In general, electron microscopy is of use only in retrospective examination of confirmed positive fluids or biopsy material. Ultrastructural changes are most prominent in percutaneous liver biopsy specimens (Winn et al., 1975). Both whole virions and viral antigens are more frequently seen in cells with a normal or near-normal ultrastructure, yet virus is rarely observed in areas of extensive hepatocellular necrosis. These changes are almost certainly due to the direct cytocidal action of the virus, as inflammatory reactions are minimal with polymorphonuclear lymphocytes and monocytes being few in number and restricted to the sinusoids or the periphery of zones.

Complement fixation (CF) methods have been widely used for the detection of arenavirus antibodies and provided a basis for establishing the extent of serological relatedness between members of the arenaviridae (Casals, 1975). Development of CF antibodies between early and late samples of sera using inactivated antigen prepared from infected suckling mouse brain is of some diagnostic value, but the limited sensitivity of this procedure makes this unsuitable for early diagnosis since antibodies do not appear in Lassa infection until the third or fourth week following onset of illness (Buckley and Casals, 1970). The preparation of a suitable CF antigen from infected cells has been described in detail by Tomori (1980). Frozen and thawed extracts of virus-infected cell cultures were inactivated by addition of beta-propiolactone at a final concentration of 10^{-4}M and held at pH 9.3 for up to 20 h. Subsequent freeze-drying of the extract produced an antigen source suitable for shipment to other laboratories as a diagnostic reagent. The material remained stable in a freeze-dried state for up to one month at 4°C, providing the CF antigen was resuspended into buffer containing bovine albumin.

Antibodies against Lassa virus may be detected by immunofluorescence in acute infection from 7 days after onset of illness (Wulff and Lange, 1975) and this is currently the method of choice for the detection

of specific antibody. Optimum sensitivity is achieved by the use of the indirect method and specific reactions characterized by the appearance of stained cytoplasmic aggregates of viral antigens, using cell cultures previously fixed by immersion in acetone. The fixed cell substrates may be stored at –70° for many months without any significant deterioration in quality.

These studies have shown that viral antibodies may be detected in serum even during the high level of viraemia found in acute Lassa infection. Immunofluorescence may also be used for the titration of specific antibody and such studies have shown that Lassa antibodies persist for at least 5 years following infection. Furthermore, endpoints do not vary significantly between substrates prepared with different virus isolates, confirming the general applicability of this method for Lassa serodiagnosis. A further advantage is the modification of immunofluorescence for the detection of Lassa antibodies of specific subclasses. Using this method, Wulff and Johnson (1979) found that IgM antibodies developed within one week after infection and rarely lasted beyond one month. Antibodies of the IgG class developed simultaneously during or immediately after the acute phase in those patients who recovered. This closely resembles the profile of antibody responses seen in experimentally infected rhesus monkeys (Fig. 3.1). Interestingly, antibodies could not be detected in the small number of sera from patients who succumbed to the infection, although a high titre of circulating virus presumably could interfere with antibody detection in such cases. Pre-treatment of sera for detection of specific IgM antibody proved necessary with some sera, although the

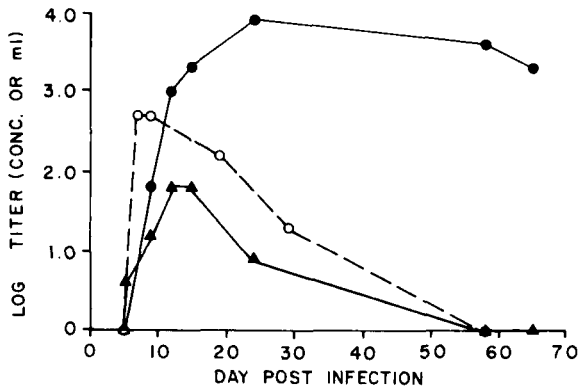

Fig. 3.1. Antibody responses in acute Lassa infection of rhesus monkeys (Walker et al., 1982b). IgG (●—●) and IgM (▲—▲) responses develop during vicaemia (O---O).

authors concluded that substances which could give rise to false positive IgM reactions are not regularly produced in Lassa virus infections.

An interesting variation in the use of immunofluorescence for rapid diagnosis and seroepidemiology has recently been proposed by Johnson et al. (1981) whereby a Lassa antigen-containing cell substrate is mixed together with cells previously exposed to the unrelated Marburg and Ebola agents. This polyvalent substrate was equally as sensitive as the monovalent antigen source for the detection of Lassa antibodies and may be of value on the African continent for rapid screening of sera for antibodies to these viruses prior to further more definitive studies.

The sensitivity of available methods for antibody detection may be improved by the use of fixed chick erythrocytes coated with Lassa antibodies. Goldwasser et al. (1980) found that up to 43% of sera collected during a survey of hospital staff from Sierra Leone gave positive haemagglutination inhibition reactions using partially purified antigen as an antigen source. This contrasted to 20% and 30% positive reactions recorded with the same panel using immunofluorescence and radioimmunoassay respectively. However, the authors point out that this test was approximately of the same sensitivity for detecting antibodies during acute infection. Although both immunofluorescence- and haemagglutination-inhibition tests avoid the problems encountered with anticomplementary sera, haemagglutination-inhibition is unsuitable for the detection of IgM virus specific antibodies.

3.2.3. Bolivian and Argentinian haemorrhagic fevers

Although the clinical features of Bolivian and Argentinian haemorrhagic fevers are very similar, the laboratory diagnosis is approached in a somewhat different manner for these viruses. Considerable information has been accumulated regarding the direct isolation of Junin virus by inoculation of guinea pigs. In an early study, Boxaca et al. (1965) found a viraemia in 22 of 23 acutely ill patients from whom serial samples were available. Virus appeared in the blood from the 2nd to the 12th day of illness, with the highest frequency of positive isolations from the 3rd to the 8th day.

Direct recovery of Machupo virus from acutely ill individuals is more difficult; infant hamsters appear to be more sensitive than suckling mice although the low titre of virus in blood during the acute phase of Bolivian haemorrhagic fever make this unreliable. Approximately 2 logs greater levels of virus may be detected in infant hamsters compared to mice (Johnson et al., 1967). In the study of Johnson and colleagues (1965b),

hamsters and mice inoculated with Machupo virus typically showed signs of illness between one and 3 weeks later. Positive isolations accounted for only 20% of 189 serial specimens obtained from 44 patients by Johnson et al. (1967). Virus was recovered from only 14 (32%) of these individuals, most commonly between the 7th and 12th day of illness. Isolations may be made from specimens of saliva or blood, but none from urine specimens, presumably because of the pH sensitivity of this virus (Webb et al., 1967). Indeed it seems likely that the virus may be transmitted by oral secretion (Douglas et al., 1965). The titres are invariably low and no definite temporal pattern can by ascertained in relation to disease development. Virus can be more readily recovered from the spleen and lymph nodes of fatal cases, with titres as high as 10^6. Splenic tissues obtained at autopsy is a reliable means of making a specific diagnosis of Bolivian haemorrhagic fever in fatal cases. Despite frequent neurological involvement, infectious virus has not been recovered from the brains of such patients.

In cell cultures, Machupo virus forms plaques in Vero cells but, unlike Junin and Tacaribe viruses, does not induce a cytopathic effect (Simizu et al., 1965). A continuous rabbit kidney cell line (MA-111) may also be used for plaque titration (Johnson et al., 1965b; Tauraso et al., 1964), with plaque formation complete 10 days after inoculation.

CF antibodies may be detected sufficiently early in the case of both Bolivian and Argentinian haemorrhagic fevers, providing suitable paired sera are available. Although this technique has now largely been superceded by the use of more sensitive immunofluorescence methods, the appearance of CF antibodies may still provide useful information as to the course of the infection and signal an onset of convalescence. Mackenzie et al. (1965) found that CF antibodies were found on average as early as 2 weeks after the onset of symptoms in 94 patients with confirmed Bolivian haemorrhagic fever. Highest antibody titres were observed between 40 and 60 days after onset and continued to be readily detected up to 80 days. This study also included the use of either Junin or Tacaribe virus antigens in place of homologous antigen in order to compare the relative sensitivity of the latter reagents for diagnostic purposes. Mackenzie et al. found similar serological profiles substituting heterologous antigen for Machupo; CF antibody titres were approximately 2-fold less using the Junin antigen. In the case of Tacaribe virus antigen, titres were much lower, negative reactions being seen in almost half of the specimens taken later than 6 months after acute infection.

Early use of immunofluorescence techniques for the diagnosis of Argentinian haemorrhagic fever showed that specific antibodies could be

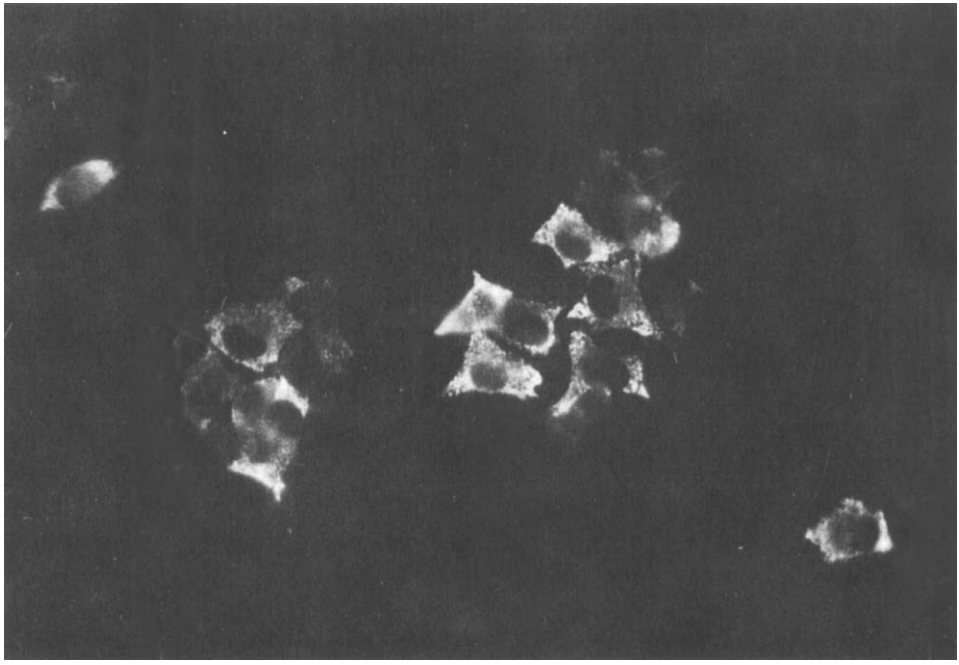

Fig. 3.2. Immunofluorescent staining of mouse L cells infected with Junin virus and reacted with antisera to the nucleocapsid. Note the intense granular fluorescence in the cytoplasm.

detected by the indirect method approximately 30 days after onset of symptoms (Zannoli et al., 1975; Grela et al., 1975). Specific staining is generally seen as a bright, granular fluorescence evenly distributed over the cytoplasm of the fixed infected cell substrate (Fig. 3.2). De Braccio et al. (1978) found specific antibodies during the acute phase of illness, albeit within a few days of the beginning of the 3rd week of illness when clinical improvement began to be evident in all 16 patients studied. The positive reaction was observed as mainly cytoplasmic and finely granular in nature using fixed infected BHK-21 cells as an antigen substrate. In a more detailed study, Cossio et al. (1979) reported that the titre of immunofluorescent antibodies increased from the 12th to the 20th day of illness. The specificity of the reactions were confirmed by prior blocking with spleen extracts prepared from infected guinea pigs. Specific immunoglobulin detected in the first positive serial specimen was determined as predominant IgG and IgM. However, in three cases IgA and IgM was detected and IgA in a further two patients. This finding of specific IgA has not been confirmed by other workers, but is of interest considering the tropism for cells of the lymphatic system exhibited by Junin virus. It has

been suggested by Carballal et al. (1980b) that persistently infected BHK-21 cell cultures offer certain advantages as a source of viral antigen, and demonstrated that this substitution into the diagnostic procedure for detecting immunofluorescent antibodies was preferable owing to the consistent number of cells positive for viral antigen at any one passage level. Identical results were obtained on a panel of sera from 32 Argentinian haemorrhagic fever patients as compared to the standard procedure using an acutely infected cell preparation as a substrate.

Peters et al. (1973) examined in some detail the titre of antibodies present against Machupo antigens in patients with Bolivian haemorrhagic fever. Interestingly, the sensitivity of the indirect immunofluorescence procedure could be improved 2-fold by increasing the incubation time to as long as 18 h at 4°C, although there was some increase in background staining. This would suggest that human antibodies to this virus may be of comparatively low affinity. Paired sera were available for 40 patients and almost all showed a 2-fold or greater rise in titre as convalescence developed. Titres were found to be highest 4–5 weeks after infection, thereafter gradually declining in parallel with the levels of neutralizing antibody.

Neutralizing antibodies to both Machupo and Junin viruses persist for many years at high titre. Webb et al. (1969) described neutralizing antibodies as appearing simultaneously with CF antibodies in more than 30 cases of Bolivian haemorrhagic fever; however, CF titres dropped significantly over the first 12 months of convalescence and only 55% were positive. In contrast, all remained positive for neutralizing antibody over this same period. Follow-up studies of patients involved in several epidemics of Argentinian haemorrhagic fever have similarly shown that CF antibodies to Junin virus are comparatively short-lived and therefore indicative of recent or ongoing infections.

The sensitivity and specificity of neutralization tests for detecting immunity to Junin virus has proven to be of value in the detection retrospectively of subclinically infected individuals (Teyssie et al., 1971; Weissenbacher et al., 1976a, 1978b, 1980b). The test may be carried out in Vero cell monolayers with the use of the varying virus dilution-constant serum method. Antibody titres are then expressed as an index calculated by subtracting the logarithmic difference between the virus titre in control and experimental reactions. Weissenbacher et al. (1978b) point out that sera giving indices greater than 1.7 are regarded as being positive; sera giving intermediate values in the range of 1.0 to 1.7 are only reported as positive if similar results are obtained on at least two further neutralization tests. Inapparent infections have been shown in approximately

20% of laboratory workers handling known or presumptively positive specimens.

Maiztegui and colleagues have drawn attention to the co-existence of LCM in natural rodent populations within the endemic areas of Argentinian haemorrhagic fever (Maiztegui et al., 1972; Sabattini et al., 1974). Junin virus was isolated from two wild-caught *Calomys musculinus* rodents at the same time as the recovery of LCM virus from *Mus musculus* trapped in the same area. Also LCM virus has been found in several patients thought to be recovering from the disease (Barrera Oro et al., 1970; Maiztegui, 1975). Antibodies to LCM were detected in these patients owing to quantities of LCM viral antigen contaminating the Junin antigen reagent used for serology. Further analysis of nearly 3000 suspected cases of Argentinian haemorrhagic fever was performed in order to dissect these immune responses to unrelated arena viruses (Barrera Oro et al., 1977). Three types of positive responses were found: the first consisted of seroconversion to Junin virus only, the second to LCM virus only, and the third group contained antibodies to both viruses. In the latter case, possible prior infection with LCM virus may account for simultaneous antibody to the second virus, particularly as Junin virus was successfully isolated in the acute phase of haemorrhagic fever in two patients, despite the concomitant presence of neutralizing antibodies to LCM virus. However, it is likely that patients showing exclusive seroconversion to LCM virus were in fact probably infected with LCM virus although clinically diagnosed as cases of Argentinian haemorrhagic fever. Taken together, this study estimated that 0.2% of Argentinian haemorrhagic fever were in fact cases of lymphocytic choriomeningitis, and that over 5% of all acutely ill patients have some serological evidence of exposure to LCM virus. These prior infections may have been comparatively recent as both CF and immunofluorescent antibodies are known to be relatively short-lived in patients convalescing from arenavirus infections.

In the 1960s, Gianantonio et al. (1964, 1968) described several new arenavirus isolates from infants with haemolytic uraemia syndrome. These viruses were collectively referred to as Portillo virus. However, Mettler and Casals (1973) conclusively showed that this new agent induced neutralizing antibodies which cross-reacted completely with Junin virus. The specificity of the neutralization test for identifying and classifying arenaviruses further suggests that Portillo virus is in fact another strain of Junin virus. This is supported by the similar paralytic sequelae of both these viruses after inoculation into newborn mice.

3.3. Serological cross-reactivity between arenaviruses

3.3.1. Studies using polyclonal antisera

Each member of the arenavirus family possesses some antigenic relationship to other members of the group, although the degree of cross-reactivity largely depends on the assay system used. The CF test reveals the broadest relationships (Casals, 1975), although it should be mentioned that early indications of Tacaribe and LCM virus belonging to the same virus group were obtained by immunofluorescence analysis of animal antisera against heterologous antigen substrates and that only limited cross-reactions were observed by CF (Rowe et al., 1970a). As with all serological tests the potency of the antigen and antisera together with the species immunized to prepare hyperimmune reagents will determine the sensitivity of such methods. Qualitative determinations between different laboratories using separate reagents are thus difficult to compare.

Serological cross-reactions in the immunofluorescent test can also be detected in patients infected with Bolivian haemorrhagic fever (Peters et al., 1973). Several specimens showed that antibody titres against substrates prepared with other members of the Tacaribe complex were highest during early convalescence and decreased thereafter in direct proportion to the titre obtained with the homologous, Machupo-infected substrate. Greatest cross-reactivity was seen with Junin antigens and at a level approximately the same as that seen using Machupo substrates for the assessment of antibody in patients convalescing from Argentinian haemorrhagic fever. Other cross-reactions were against Tacaribe and Latino viral antigens; perhaps surprisingly positive results were also observed using an LCM virus-infected substrate. It is relevant to note that acetone-fixed substrates give predominantly cytoplasmic reactions.

Much of the antigen load within infected cells represents nucleocapsid antigens rather than the type-specific envelope proteins. Antibody to these are best detected by examining for immunofluorescence at the plasma membrane of unfixed cells mixed with antisera in suspension.

A particularly strong relationship between Tacaribe, Junin and Machupo viruses can be readily demonstrated by CF with a more distant cross-reactivity discernible between these viruses and other members of the Tacaribe complex, although Pichinde and Tamiami are not so closely related to each other or to other New World arenaviruses (Casals et al., 1975). All the available evidence suggests that the complement-fixing antigen is associated with the internal nucleoprotein; studies by

Gschwender and Lehmann-Grube (1973) determined that this activity was associated with a soluble fraction obtained from LCM infected cells and that repeated inoculations of animals resulted in antisera with a high titre by the CF test but the same antibody preparation failed to neutralize virus infectivity. Moreover, guinea pigs inoculated with the soluble antigen only were not protected against subsequent challenge with virus. Using the CF test, LCM and Lassa viruses show some relationship to each other; however, these cross-reactions are now being more satisfactorily resolved by taking advantage of the monospecifity of monoclonal antibodies.

3.3.2. Studies using monoclonal antibodies

The availability of monoclonal antibodies to several arenaviruses in recent years has permitted a greater analysis of the fine specificity differences between arenavirus antigens. For example, Buchmeier et al. (1980b) characterized 46 monoclonal reagents raised against the Armstrong CA-1371 strain of LCM virus. Cross-reactivity was detected with five antibodies by indirect immunofluorescence using cells infected with Lassa or Mopeia viruses and a sixth reacted with the Mopeia virus only. Interestingly, this reagent specific for LCM and Mopeia viruses only recognized the external G2 glycoprotein (see Chapter 7) of LCM virus whereas the remaining antibodies additionally recognized Lassa-infected cell substrates were directed against epitopes on the internal nucleocapsid of the LCM virion. A more recent report from the same laboratory has shown that monoclonal antibodies to the G2 glycoprotein of the WE strain of LCM virus also may recognize Mopeia but not Lassa viral antigens. A further four antibodies specific for the large G1 glycoprotein of LCM virus did not cross-react with either of the African arenaviruses (Buchmeier, 1984). These results suggest that there is a degree of conservation of epitopes on the G2 molecule and the finding of cross-reactivity with these reagents also against Pichinde virus of the New World suggests that this conservation is not restricted to the Old World arenaviruses. Direct confirmation of the immunofluorescence findings has been obtained by immunoprecipitation of viral proteins from infected cells and analysis of the immunecomplexes by SDS-polyacrylamide gel electrophoresis (Buchmeier et al., 1981; Kiley et al., 1981; Buchmeier, 1984). A problem often encountered in these studies, however, is the use of *Staphylococcus aureus* protein A (Kessler, 1976) for the collection of antigen-antibody complexes. This may result in the non-specific binding of nucleocapsid polypeptide (Kiley et al., 1981; Howard et al., 1985).

Monoclonal antibodies have also been prepared against members of the Tacaribe complex of New World arenaviruses, the vast majority being specific for epitopes of the internal nucleocapsid antigens. Antibodies to Pichinde virus frequently cross-react with other members of the complex and in one instance cross-reactions have been recorded against Lassa viral antigens, suggesting that at least some epitopes situated on the internal nucleocapsid may be shared by both New and Old World arenaviruses (Buchmeier et al., 1981). In the latter study, the extent of cross-reactivity differed between antibodies examined, suggesting that each recognized a unique determinant. Similar findings have also been reported with a collection of monoclonal antibodies prepared against Tacaribe virus (Allison et al., 1984; Howard et al., 1985). Among those reagents specific for the nucleocapsid of Pichinde virus, a particularly close relationship could be discerned between Pichinde, Parana and Tamiami viruses with other cross-reactions seen using Amapari and Junin substrates. Antibodies to Tacaribe and Junin viruses have confirmed the particularly close relationship between these two viruses and Amapari and Machupo viruses. Pichinde and Parana viruses appear to be more distantly related (Allison et al., 1984; Howard et al., 1985). These observations are consistent with previous findings using hyperimmune antisera, although more antibodies to all members of the Tacaribe virus complex are required to more fully understand the evolutionary relationships among the New World arenaviruses. A particularly fascinating close antigenic relationship exists between the envelope glycoproteins of Tacaribe and Junin viruses. For example, both guinea pigs and marmoset monkeys inoculated with Tacaribe virus are protected against challenge with the normally lethal Junin virus (Weissenbacher et al., 1975/76, 1982). Yet these viruses were originally isolated from regions some 3000 miles apart and from totally different natural host species. The availability of monoclonal antibodies to the envelope glycoproteins of these viruses together with rapid advances in genome sequencing techniques should reveal the immunological basis of this cross-protection and the nature of the antigens involved.

Monoclonal antibodies are increasingly used to differentiate virus strains by use of reagents selective for epitopes which go unrecognized when polyclonal antisera are used. Buchmeier (1984) has summarised these patterns of reactivity obtained with a panel of monoclonal antibodies against laboratory strains of the homologous virus and also Lassa and Mopeia viruses. Reagents directed against the smaller, G2 envelope glycoprotein were found to cross-react by immunofluorescence with all substrates examined, whereas antibodies directed against the larger G1 gly-

coprotein (see Table 7.3., page 129) were either strain-specific or reacted with only a subset of the different strains examined, presumably by binding to previously unrecognized epitopes. The observation that certain of these broadly cross-reactive antibodies also reacted with Pichinde virus suggests the conservation of epitopes on surface envelope structures among Old World and New World arenaviruses. A similar type of comparison has also been undertaken using monoclonal antibodies to Lassa and the Mopeia and Mobala viruses from Africa (Gonzalez et al., 1983, 1984b). Again various degrees of cross-reactivity were observed using reagents specific for the G2 external glycoprotein. Some distinction may be made with Mobala virus from the Central African Republic, however, as it was found that several cross-reactive monoclonal antibodies originally prepared against LCM virus failed to recognize Mobala-infected substrates.

Clegg and Lloyd (1984) have analyzed an extensive range of different Lassa and Mopeia strains and found antigenic determinants common to all strains of both viruses on the internal nucleocapsid and on at least one of the two envelope glycoproteins. Interestingly, sera raised against Mopeia virus showed a pattern of reactivity restricted to the nucleocapsid of this virus with no reactivity against Lassa virus.

Although the introduction of monospecific antibodies using hybridoma techniques has begun to unravel the antigenic complexity of arenaviruses, the preparation of more reagents is required to make many of these comparisons meaningful, this is hampered somewhat by the difficulty in preparing monoclonal antibodies specific for the envelope virion glycoproteins where the greatest antigenic diversity may be expected. Antibodies prepared against other arenaviruses would also be useful for reciprocal comparisons of serological cross-reactivity.

3.4. Virus neutralization

The neutralization test is very specific for all members of the arenavirus family, with only limited examples of cross-reactivity, notably using high titre animal antisera raised against Junin, Tacaribe and Machupo viruses. However, the ease of quantifying neutralizing antibodies in the laboratory differs sharply. These difficulties are summarized in Table 3.4. No cross-reactions have been observed between Junin and Machupo viruses in plaque-reduction tests using human convalescent sera despite the close relationship that can be demonstrated by CF. A similar marked specificity of neutralization has been demonstrated with LCM and Lassa

TABLE 3.4.
Ease of detection *in vitro* of neutralizing antibodies to arenaviruses

Old World	New World
LCM[a]	Tacaribe[c]
Lassa[b]	Machupo[c]
Mopeia[b]	Junin[c]
	Pichinde[b]
	Tamiami[b]
	Amapari[b]

[a] Neutralization requires the addition of complement.
[b] Neutralization detected only with difficulty, often less than 1 log of virus.
[c] Neutralization readily demonstrated, often greater than 1 log of virus.

sera and both viruses are readily distinguishable from one another by this technique. The sensitivity of the neutralization test for LCM virus can be increased by incorporating either complement or anti-gammaglobulin into the test system (Oldstone, 1975; Lehmann-Grube and Ambrassat, 1977). However, neutralizing antibodies to Lassa virus can only be detected with great difficulty.

It is well recognised that high titred antisera against either Pichinde or Lassa viruses possess minimal neutralizing capacity when examined by the mouse neutralization test (Lehmann-Grube et al., 1979) or by tissue culture tests (Buckley and Casals, 1970; Sengupta and Rawls, 1979). The use of an alternative and simple method for detecting antibodies to Pichinde virus has been described whereby the incorporation of antiserum into the overlay of an infected cell culture results in plaque-size reduction (Chanas et al., 1980). Such antisera may be deficient in antibodies directed against an essential virus surface component with the result that the covering of critical areas required for virus neutralization does not occur. Whether this may be the case in convalescent sera to Lassa virus is unclear, but the studies of Chanas et al. showed clearly that aggregation and extensive sensitization of Pichinde virus particles does occur after reaction with homologous antiserum. Despite the presence of antibodies directed against all the major structural proteins of the virus, absorption and penetration of virus antibody complexes were not prevented.

Buchmeier (1984) has reported neutralization of LCM virus by monoclonal antibodies in the absence of complement. One of the three antibodies studied neutralized both the Armstrong CA-1371 and WE strains, demonstrating that there may be limited sharing of epitopes involved in

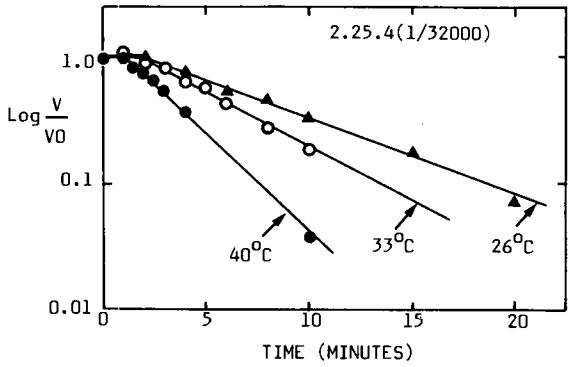

Fig. 3.3. Kinetic neutralization of Tacaribe virus with monoclonal antibody 2.25.4 at various temperatures. Reduction in the titre of infectious virus is expressed as the $\log_{10}$ value of residual virus (V) divided by the virus titre at time 0 (V0).

the relevant antigenic site between closely related viruses. These antibodies all react with the larger, G1, envelope glycoprotein of the virion, a result in agreement with the observations of Bruns et al. (1983b) who purified this protein from virus by affinity chromatography using an immobilized monoclonal antibody with neutralizing activity.

Similar analysis of arenavirus neutralization by monoclonal antibodies in the absence of complement has been reported using Tacaribe virus (Allison et al., 1984; Howard et al., 1985). Five monoclonal antibodies directed against determinants on the outer viral envelope were found to neutralize infectivity at high dilution, although no reactions were recorded against the closely related Junin virus. This is in contrast with the one-way cross-neutralization observed using polyclonal mouse antisera as reported by Henderson and Downs (1965); in the latter instance, anti-Junin sera failed to neutralize Tacaribe virus whilst anti-Tacaribe immune ascites neutralized both Junin and the homologous virus.

Antibodies against Tacaribe virus positive for neutralization have been examined in order to quantitate the rate of reaction between antibody and virus (Allison et al., 1984). Kinetic neutralization experiments showed that antibodies differed according to the level of infectivity remaining after 20 min of incubation. At high dilution, one antibody (see Fig. 3.3) neutralized all infectivity after an initial lag period, suggesting that two or more molecules of antibody are required for neutralization as single hit theory assumes a linear response from the time of mixing virus with antibody (Della Porta and Westaway, 1978). The remaining anti-

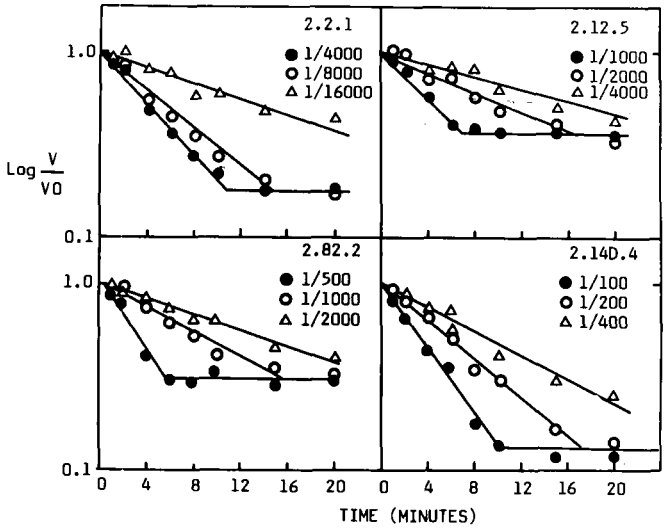

Fig. 3.4. Kinetic neutralization of Tacaribe virus with four monoclonal antibodies directed against the surface glycoprotein but producing a large resistant fraction. Dilutions of antibody examined are shown adjacent to symbols. Reduction in infectivity is expressed as the $\log_{10}$ value of residual virus (V) divided by the virus titre at time 0 (V0) (from Howard et al., 1975).

bodies gave significant non-neutralized fractions after an initial linear response (Fig. 3.4). Residual virus infectivity was decreased by addition of either antibody or anti-mouse IgG to the virus-antibody mixtures, and a similar reduction was also observed with fresh complement (Howard et al., 1985). Given the monoclonal nature of the antibodies used in these studies it is unlikely that the persistent fraction is due to steric hindrance from non-neutralizing antibodies. One alternative explanation is that such large persistent fractions may indicate incomplete neutralization as a result of an equilibrium between free and bound antibody molecules at a point prior to complete neutralization (Volk et al., 1982). However, a recent study using similar methods to quantitate Newcastle Disease Virus neutralization with monoclonal antibodies suggests that lower avidity does not necessarily result in an increase in the size of the non-neutralized fraction (Iorio and Bratt, 1984). This latter study did show, however, that a mixture of monoclonal antibodies directed against four distinct epitopes on the virus surface showed neutralization kinetics similar to those obtained with polyclonal animal antisera. A similar situation may be the case for Tacaribe virus in that monoclonal antibodies directed against other, hitherto unidentified sites may singly or in combination with other epitopes constitute important domains for eliciting neutralizing antibodies.

Some insight into arenavirus neutralization may be gained by the selection of virus variants grown in the presence of neutralizing monoclonal antibody. Howard et al. (1985) have reported that Tacaribe virus variants may be selected in the presence of antibody at a frequency greater than 10^{-4}. Reaction of the variants with heterologous monoclonal antibodies manifesting incomplete neutralization suggests that point changes occurring in or around the epitope recognized by the selecting antibody may alter the configuration of non-overlapping distinct epitopes recognized by the second subset of antibodies. Such mutations of the viral genome may not necessarily result in alterations of any one particular amino acid of the translated sequence, however, and the nature of these changes may have variable effects on the ability of the glycoprotein to bind other monoclonal antibodies.

CHAPTER 4

Human arenavirus infections

Arenavirus infections of man vary in clinical presentation. Although the term haemorrhagic fever is often applied to all but LCM virus infections, each of the four diseases described in this chapter show distinct clinical signs which reflect differences in tissue tropism and host responses to these viruses. Almost certainly there are many more cases of human infections than have hitherto been recognized, although there is a heightened awareness among infectious disease specialists in the northern hemisphere of cases originating in West Africa and to some extent, various regions of the South American continent. Perhaps LCM infections may go unrecognized, as this frequently presents as an influenza-like syndrome. Also this is the only arenavirus pathogen known to persistently infect peridomestic rodents in North America and Western Europe. The neurotropism of LCM virus is also shared by the agents of Bolivian and Argentinian haemorrhagic fevers, whereas the pathology of Lassa fever is notable for the involvement of the liver.

Much is known about the epidemiology of haemorrhagic fevers, many of which have come to light only during the last 30 years. Johnson (1982) has suggested that population increases and the continuing incursion of man into new ecosystems has played a major role in bringing these diseases to the attention of the medical world. The global use of antibiotics and antimalarial compounds has also revealed many acute febrile infections of non-bacterial origin, although it is now unlikely that many new arenavirus-induced syndromes remain to be discovered. This does not preclude, however, the finding of new arenaviruses which solely cause

persistent infections of rodents in parts of the world other than the American and African continents.

4.1. Lymphocytic choriomeningitis

LCM virus was originally described by Armstrong and Lillie (1934) as a result of the pathology induced in monkeys inoculated with a brain tissue extract taken from a fatal case of suspected St. Louis encephalitis. Rivers and Scott (1935) subsequently produced evidence establishing an aetiological relationship between LCM virus and acute aseptic meningitis in man previously described by Wallgren (1925). The latter syndrome was described in detail as being characterized by an acute onset of meningitis accompanied by an increase in the number of mononuclear cells in the cerebrospinal fluid. The disease was relatively short in duration with no evidence of a causal relationship with any other pathogen known to cause meningitis. Although human LCM virus infection is still invariably linked to the term aseptic acute meningitis, it has been stressed by Lehmann-Grube (1971) that the original syndrome described by Wallgren may have a multiple aetiology of which the virus may only be one factor.

Human LCM virus infection may occur world-wide, with cases having been recorded from both North and South America, Asia and Europe. It usually occurs in persons between the ages of 15 and 40 years, there being no difference in susceptibility between males and females. Armstrong (1941) showed that approximately 11% of a random population in the United States possessed antibodies although more recent studies show that this figure may be much less. However, a retrospective survey in the U.S.A. over a 4-month period in the winter of 1973–1974 uncovered 181 laboratory confirmed cases (Gregg, 1975). Infection seems to originate either by natural exposure to LCM virus shed from infected rodents or arising from laboratory accidents. An exhaustive list of relevant papers prior to 1971 on the epidemiology of LCM virus is to be found in Lehmann-Grube (1971). Oldstone and Peters (1978) distinguished four broad categories of clinical disease: (1) inapparent or subclinical infection; (2) a nonmeningeal illnes resembling influenza; (3) aseptic meningitis and (4) meningoencephalomyelitis. Even though estimates as to the prevalence of naturally acquired antibodies may vary, it is clear that the virus most commonly produces either a subclinical infection or a mild febrile illness that is often described as being influenza-like.

There are several reports of grippe-like illness which have subsequently

been confirmed as due to LCM virus (Baum et al., 1966; Vanzee, 1975). These individuals present with fever, malaise and muscular pain after an incubation period of between 1 and 2 weeks. A coryza together with retro-orbital pain, anorexia and nausea were common findings. In the group of 10 male patients studied by Baum et al., there were no signs of meningitis and the cerebrospinal fluid appeared normal. In the second group of 15 patients studied by Vanzee and colleagues, only two showed overt meningitis. During convalescence, however, unilateral orchitis developed in a minority of patients. Both parotitis and orchitis has been estimated as occurring in about 10% of cases (Lewin and Utz, 1961). It should be noted, however, that in both these studies infection arose as a result of prior virus passage in a rodent unrelated to its natural host.

The neurological signs accompanying human LCM virus infection have been detailed by Oldstone and Peters (1978). The aseptic meningitis form is associated with a febrile illness, anorexia, headache, malaise, irritability and a pronounced stiffening of the neck (Scott and Rivers 1936; Farmer and Janeway, 1942). During the acute phase a large number of mononuclear cells are present in the cerebrospinal fluid as part of a pleocytoses, although the absolute number varies according to the time after onset of disease. Generally, the mean value of mononuclear cells is approximately 600 cells per mm^3 although counts of up to 3000 per mm^3 have been recorded. It has been noted that the majority of such patients have a history of influenza-like illness immediately prior to the onset of meningitis.

The most severe form of infection is characterized by a meningoencephalitis, which may immediately follow the acute meningeal phase. Findlay and Stern (1936) described two such patients who rapidly developed a bilateral papilloedema confusion, hallucinations and paralysis of the extremities over a period of one week.

4.2. Lassa fever

4.2.1. Epidemiology and clinical diagnosis

Acute infection with Lassa virus is characterized by an unremitting febrile illness with temperatures of 100°F or higher. Although the clinical signs may be confused with yellow fever, malaria or typhoid, Lassa fever must be suspected if temperatures remain elevated for one week or more accompanied by pharyngitis, malaise, leucopenia, and unresponsiveness to antibiotics or anti-malarial drugs (Monath, 1973). Despite

TABLE 4.1.
Symptoms associated with probable past Lassa infection as suggested by Fraser et al. (1974)

Major criteria	*Minor criteria*
pharyngitis	headache
abnormal bleeding	myalgia
swelling of the face or neck	abdominal or chest pain
leucopenia (less than 4000 per mm^3)	nausea or vomiting
systolic blood pressure less than 100 mm/Hg and pulse pressure less than 20 mm/Hg	cough
	lymphadenopathy
alopecia within 2 months of illness	
pleural effusion or ascites	
death on or after 10th day of illness	

the highly contagious nature of the infection, good clinical descriptions exist of acutely ill patients hospitalized either in endemic areas or transported to the northern hemisphere for intensive treatment. Although some similarities exist with individuals acutely infected with the South American arenaviruses (see Table 4.1), the course of Lassa fever is much more severe resulting in a high mortality rate. Tropism of the liver and very high levels of circulating virus may be factors, although it is clear that there is a spectrum of disease associated with Lassa virus in man from a mild, almost asymptomatic condition to the serious and often fatal haemorrhagic illness.

The events surrounding the initial emergence of Lassa fever in January 1969 have been dramatically reconstructed in the book *'Fever'* by Fuller (1974) and in the scientific press by Frame et al. (1970). The first reported case was a 69-year-old nurse working at a remote missionary hospital established in the 1920s to provide basic medical care facilities around the small town of Lassa situated close to the Cameroon highlands in the far north-east of Nigeria (Fig. 4.1). One week after complaining of backache and malaise the patient developed a severe sore throat and high temperature. Small ulcerations in the pharynx and buccal mucosa rapidly increased in number over several days and developed as lesions with yellowish centres surrounded by erythematous halos. Further symptoms included signs of dehydration, facial oedema, a macular and petechial rash and signs of haemorrhage. This patients was subsequently evacuated to the Bingham Memorial Hospital in Jos, Nigeria, where she died of cardiac failure within 2 days of admission. Two further cases were associated with this initial outbreak. The first of these was a nurse who treated the index case soon after arrival in Jos by swabbing the throat of the patient with a gauze dressing. This was held around a finger contain-

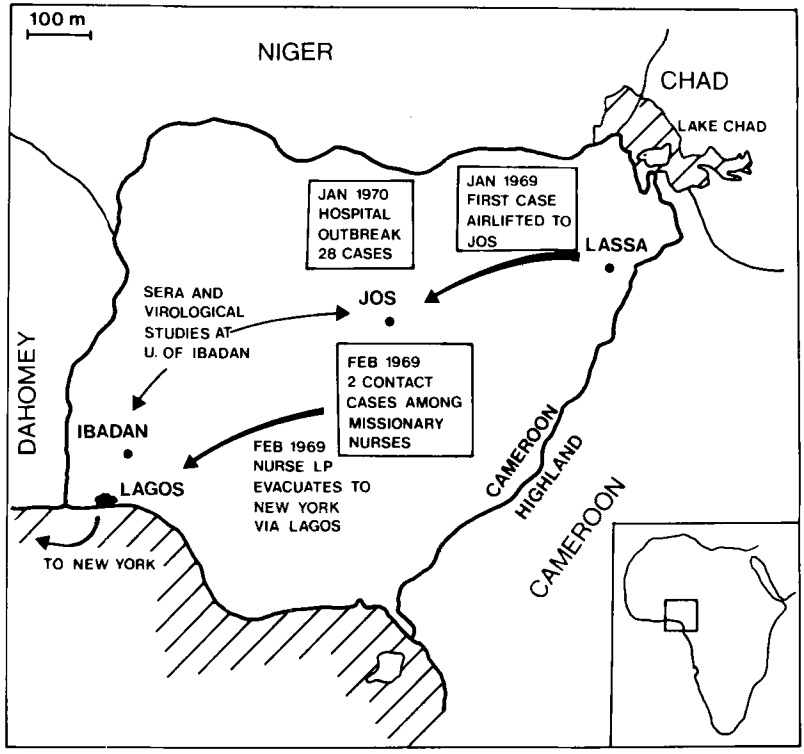

Fig. 4.1. Map of Nigeria showing the localities of the 1969 and 1970 outbreaks of Lassa fever.

ing a small cut inflicted a few hours previously. Symptoms of the disease developed 10 days later, starting insidiously at first with backache and neck pains, but rapidly developed into a severe febrile illness with a temperature over 104° F and an extensive macular rash on the face, trunk and limbs. As with the first patient, renal clearance was impaired in the later stages of the illness. This patient died on day 11 of her illness and an autopsy revealed extensive haemorrhage of the lungs, kidney and liver with ascites and fluid in the pleural cavity. A second nurse who assisted at the autopsy and previously had nursed both patients also developed Lassa fever, but recovered from the illness after having been flown to New York for treatment. During convalescence this patient was extremely lethargic and an electroencephalogram indicated a diffuse encephalopathy. Occasional rapid eye movements and a tendency to walk towards the right were reported, together with loss of hair. Irreversible loss or impairment of hearing has since been reported in patients surviving acute Lassa infection.

Frame et al. discussed the problem of diagnosis prior to subsequent knowledge of virus isolation. Although all three cases presented with similar clinical symptoms, initially other infections common to West Africa could not be excluded. Tests were therefore performed on the patient hospitalized in New York for a variety of other pathogens, including typhus, *Salmonella* and *Leptospira*, all of which proved negative. Buckley and Casals (1978) have pointed out that typhoid fever in many ways resembles Lassa fever, being an acute generalized infection characterized by pyrexia, leucopenia and involvement of the lymphatic tissues. Unlike Lassa infection, however, patients with typhoid often present with splenomegaly and respond favourably with chloramphenicol. Among possible protozoan pathogens, *Plasmodium falciparum* infection is perhaps the most likely cause of febrile illness in patients from West Africa and tests to exclude malaria are always of prime importance in initial diagnosis of suspected Lassa patients, particularly in less severe cases.

There is now extensive documentation that Lassa infection of children often follows an inapparent or milder course. For example, Henderson et al. (1972) described a child of a missionary from Guinea in West Africa who developed antibody to the virus without signs of clinical illness. Indeed, there is now extensive evidence that Lassa virus infections are common in the endemic regions of Africa (reviewed by Frame, 1975), and indeed a further outbreak of Lassa fever in January, 1970, showed that among a total of 28 cases the mortality rate was less than 40%. In retrospect, it was discovered that the index case was a young mother from a village close to Jos who had been admitted to hospital some time previously with respiratory difficulties and fever. Yellow fever was suspected at the time but the patient was subsequently discharged after a stay of 10 days. Shortly thereafter two further cases developed in a patient and a mother caring for a child in the same ward, forming the focus of a subsequent hospital outbreak. In contrast to the cases seen almost a year previously, all were among Nigerians, save for Dr. Jeanette Troub who fatally contracted the disease whilst performing an autopsy on a ward attendant who had died from the infection. The paper by Troub et al. (1970) notes that '. . . She died in line of duty, trying to gain insight into the disease that she had been primarily responsible for bringing to the attention of the medical world'.

The distinction between fatal and non-fatal cases was also clear in the first reported outbreak outside Nigeria (Mertens et al., 1973; Monath et al., 1973). Here, the index case was a pregnant woman admitted to hospital in Zorzor, Liberia, who underwent a Caesarian section whilst febrile and was suffering from respiratory difficulties. Ten secondary cases

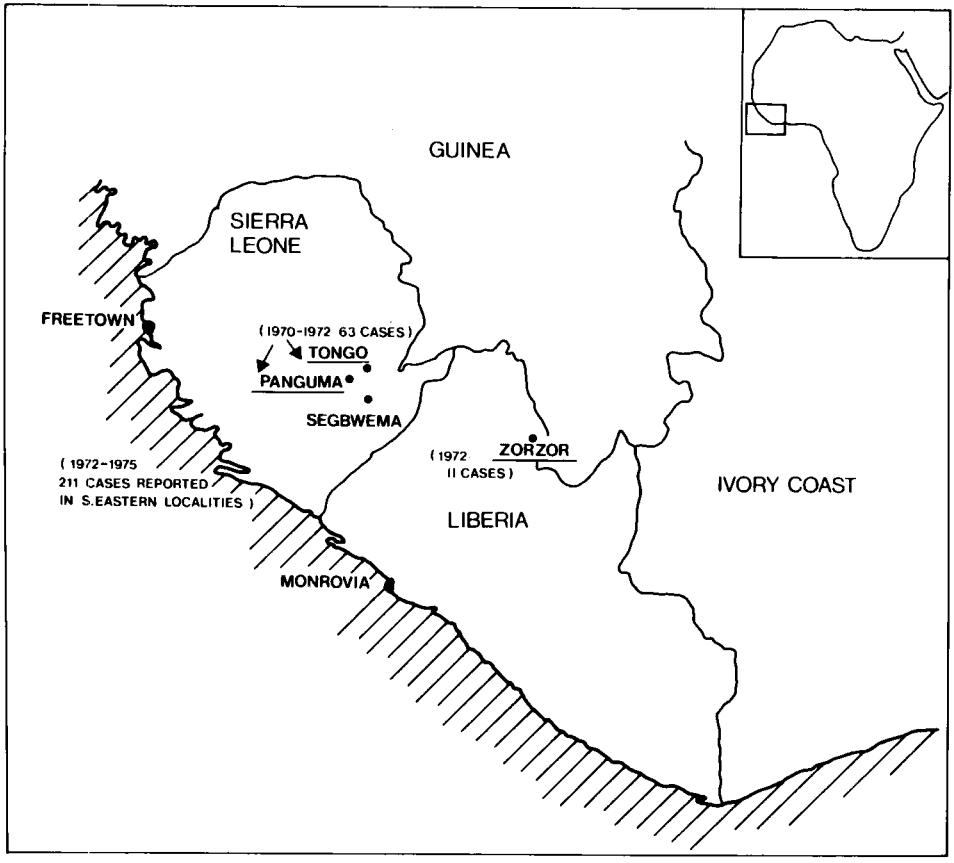

Fig. 4.2. West African countries where Lassa virus is endemic. Centres of early documented outbreaks are underlined and the year given in parenthesis. Prevalence of antibody among the general population varies from less than 5% in coastal areas to over 14% inland.

resulted, with four deaths. Among the latter group facial and neck oedema together with a macular rash were particularly noticeable and in general the clinical features closely resembled those seen previously in the two Nigerian outbreaks; pharyngitis was seen in more than 70% of the patients and nearly all presented with fever, headache and myalgia. Mertens et al. make the point that clinical diagnosis is made somewhat easier if there are a number of patients with a similar syndrome, but isolated cases are more difficult requiring access to suitable laboratory facilities for rapid diagnosis. Recently developed techniques, e.g. immunofluorescent analysis of specific antibodies in serum, have considerably

improved the situation together with the establishment of specialized laboratories for handling specimens located in endemic regions.

Awareness of possible infection together with adoption of simple quarantine procedures for febrile patients are the goals of public health authorities in such countries. As with the previously recorded outbreaks, there were few cases of tertiary clinical infections and Monath (1973) has pointed out that not all persons with severe clinical infections are capable of transmitting the virus.

Subsequent to this outbreak, a further epidemic occurred in the eastern province of Sierra Leone (Fig. 4.2), the epidemiology of which differed in several important respects. Firstly, the outbreak began in the community rather than in a nosocomial setting although hospital staff remained at high risk of infection. Secondly, several generations of cases were observed over 2 years with some evidence to suggest that transmission occurred between individuals. The epidemiology of this outbreak has been described in detail by Fraser et al. (1974) and is important, illustrating that Lassa infection is a major public health problem today in West Africa. The Sierra Leone outbreak was centred around the town of Panguma, an area important for the mining of diamonds. Retrospective examination of records in four separate hospitals showed 63 suspect cases of Lassa infection when applying the criteria outlined in Table 4.1. A probable case was defined by the recording of at least two major criteria or one major and two minor criteria; the remainder were considered possible suspects. Of these cases, only 10 (16%) were observed during the epidemic investigation, almost all of which were confirmed by diagnostic techniques as being due to specific virus infection. The attack rate in the communities examined was 1.6–4.4 cases per 1000 individuals. Among those two-thirds of suspected cases considered as probable exposures, the majority possessed demonstrable antibody to the virus. The relatively insensitive technique of complement fixation in use at the time may have missed some cases, however; although no seroconversions were found in those cases not meeting the full criteria of probable infection, direct virus isolation was achieved in two cases. The overall fatality rate in the 63 cases examined had been 30%. The preponderance of hospitalized female cases in neighbouring Liberia has been noted by Monson et al. (1984) although antibody prevalence figures for the general population show no difference in attack rates between males and females (Yalley-Ongunro et al., 1984). This may simply reflect the larger numbers of women in these communities as men often move towards larger population centres in order to find employment.

Importantly, evidence of infection was seen among staff at the Pangu-

ma Hospital; among six of a total of 24 nurses, four had been hospitalized, together with one laboratory technician. However, 19% of other staff were found to have specific antibody although none had been admitted for febrile illness. These data show that, although the majority of cases were the result of exposure to the virus outside of the hospital, medical care personnel remain at high risk, particularly nursing staff. However, many cases go unrecognized. In the surrounding communities, 6% were found to be antibody positive; probably less than 1 in 20 of all Lassa virus infections were fatal in this area when compared to the number of clinical cases observed during the epidemic.

Extensive serological surveys have been carried out at hospitals in adjacent Liberia. Although a total of 165 sera were positive for antibodies, 35 were from the Curran Lutheran Hospital in Zorzor close to the border where the 1972 outbreak of Lassa fever had occurred. Evidence was found for exposure to the virus among four midwives at this location. Further extensive surveys have shown that Lassa fever is endemic throughout Liberia with up to 40% prevalence of antibody in hospital staff in north-western areas, reflecting both community-acquired infections and transmission from patients (Frame et al., 1979, 1984a,b). It should be noted that Lassa virus has been found to be the largest single cause of morbidity in two hospitals in Sierra Leone, accounting for 30% of deaths on all medical wards (Johnson, 1982). Most of the infections were probably acquired at times different from the 1972 outbreak, indicating that the virus is endemic in the Zorzor area, and a further two seropositive midwives were recognized at a hospital in a similar locality.

The introduction of the more sensitive immunofluorescence assay for Lassa antibodies has resulted in an accurate assessment of human infection in various parts of West Africa. Sharp (1982) has pointed out that as many as 30% of children have been exposed to the virus by the age of 10 and that in an endemic area of Sierra Leone previous descriptions of so-called Yengema fever made in 1957 had features very similar to Lassa fever (Rose, 1956). The overall prevalence of antibody has been estimated at 26% in the study of Keenleyside et al. (1983) also carried out in the same region, the age-related rate being lowest in children under 10 (15%) and highest in adults over 50 years of age (41%).

Somewhat different results have recently been obtained in neighbouring Liberia by Yalley-Ogunro et al. (1984). Here a similar prevalence of Lassa antibodies was found in all age groups. These differences may in part be explained by the comparative brevity of antibody responses measured by immunofluorescence; in villages where infection is sporadic, antibody levels would fall to become undetectable between outbreaks

whereas a high rate of infection would result in a cumulative increase in antibody prevalence among older age groups. The Liberian study also revealed considerable variations in antibody prevalence between villages, with those located beside major routes of communication showing higher levels. This was reduced somewhat in those maintained to a higher degree of cleanliness, but in general antibody was found even in villages relatively unaffected by modern development. In contrast to other viral haemorrhagic fevers, evidence of human Lassa infection appears to be restricted to West Africa despite the isolation of Lassa-related arenaviruses from rodents trapped across the African continent. For example, surveys in Gabon, the Central African Republic and Zimbabwe have shown little evidence of hitherto undetected human infection (Ivanoff et al., 1982; Blackburn, 1982, Gonzalez et al., 1983).

Despite the difficulties in controlling the overall numbers of *Mastomys natalensis* within an infected region, Keenleyside et al. (1983) have attempted to examine the effect of reducing the peridomestic rodent population on infection rates as well as gain further information as to the mode of transmission. Immediately upon identification of a case of Lassa fever, rodent traps were set in the home of the patient and adjacent dwellings. An equal number of control households were also studied save no attempt was made to trap for rodents. Seroconversion rates among the occupants over a 5-week period did not appear to be significantly different between the two groups and appeared unrelated to the infection rates in trapped animals. Indeed, Keenleyside and colleagues found one control house had a particularly high infestation with infected animals without signs of clinical illness among its occupants and, conversely, only one animal with no evidence of virus infection was caught in the house with the greatest number of human Lassa cases. These observations imply that rodent to man is not the most important route of transmission in this setting. Although direct human transmission was a real possibility, there was no correlation between seroconversion rates and crowding. This study also showed the difficulty in effectively controlling rodent numbers in infested households and further studies are required to examine whether reduction in the numbers of *Mastomys* over a longer period would show a clearer relationship between the number of infected rodents and human disease.

4.2.2. Pathology

A comprehensive pathological study of fatal cases of Lassa fever has recently been reported by Walker et al. (1982a). Tissue was collected from

TABLE 4.2.
Correlation between chemical markers, viraemia and outcome of Lassa fever patients in Sierra Leone, 1978 – 1979[a] (from Johnson 1982)

Outcome	No.	SGOT[b]	No.	Log_{10} virus/ml
Death	41	923	46	4.1
Survival	89	171	104	2.1

[a] Parameters measured at time of hospital admission.
[b] International units/ml serum glutamic-oxaloacetic acid transaminase.

confirmed cases seen in Sierra Leone between 1976 and 1980 believed to be community-acquired infections. The histopathology was largely confined to the liver, spleen and adrenals. Splenic necrosis was evident in the marginal zone of the periarteriolar lymphocytes and mononuclear cells. In the adrenals, prominent cytoplasmic inclusions were present in cells near the junction of the zona reticularis and medulla. The acidophilic inclusions appeared mainly in adrenocortical cells and varied from 3 to 15 μm. Injury in the form of multi-focal necrosis was seen in the zona fasciculata and was often associated with focal inflammation. The pathology observed in the adrenal glands did not appear to affect more than 10% of the cells of the adrenal cortex.

Lassa virus is hepatotropic in man, and elevated serum transaminase values have been observed during acute infection (Leifer et al., 1970; Walker et al., 1982a). These may be over 30-fold in excess of normal values and there appears to be a correlation with viraemia levels and outcome of infection (Table 4.2). A detailed study of human liver obtained from a fatally infected 20-year-old woman has been reported by Winn et al. (1975). Light microscopy of tissue fixed in glutaraldehyde showed severe cell damage. Individual hepatocytes showed particular cytoplasmic pathology, often progressing to almost complete destruction of the plasma membrane and intracytoplasmic structures. Vacuolation in less severely damaged cells was accompanied by pigment accumulation giving rise to Councilman-like bodies, a distinct feature that has been noted on several occasions (Edington and White, 1972; Sarrat et al., 1972). Lipid accumulation was also seen although pronounced steatosis was absent. The nuclear changes were less marked, although the nucleoli were particularly prominent.

Hepatocellular damage is mainly centrilobular with bridging necrosis, although areas of focal degeneration are occasionally seen. However, inflammation is minimal, a feature distinct from the severe injury occa-

sionally observed in cases of human hepatitis B. Interestingly, Walker et al. (1982) described one fatal case which showed the 'ground glass' hepatocytes typical of those containing hepatitis B surface antigen; concurrent hepatitis B virus infection therefore does not appear to preclude replication of Lassa virus.

The few cells present in areas of greatest cell damage were mainly confined to monocytes and a few polymorphonuclear neutrophils at the periphery and along the liver sinusoids, although enlarged Kupffer cells were prominent.

Arenavirus particles may be seen by electron microscopy in the perisinusoidal and extracellular spaces as well as in bile canaliculi (Winn et al., 1975). Maturing virus was seen at the plasma membrane of infected hepatocytes, although the inclusion bodies typical of arenavirus infection in other cell types were not seen. At the ultrastructural level, a variety of pathological changes were found to accompany virus replication, namely margination of the chromatin increased nucleolar density and dilation of the endoplasmic reticulum. The first response of the hepatocyte to any injury is an alteration in the rough endoplasmic reticulum. As a result of dilation and disruption, ribosomes become detached, and small vesicles appear. The extreme swelling which gives rise to the so-called 'balloon-cells' prominent in other forms of viral hepatitis is noticeably absent in a Lassa-infected liver. As with the light microscopy findings, it was particularly noticeable that severely damaged cells may be adjacent to hepatocytes showing small or negligible signs of infection. It has been estimated that less than 40% of hepatocytes are involved in infected liver (Walker et al., 1982a).

The role of Kupffer cells in Lassa-associated hepatitis is unclear. All studies so far have shown little indication of virus growth in these cells. However, this may be due to the taking of biopsy material after the onset of acute illness at a time when replication of virus in these cells has subsided.

Pathological examination of other tissues obtained at necropsy does not appear to reveal specific alterations. Walker et al. (1982a) examined pancreas, ovary, uterus placenta and breast but findings were inconsistent and concluded any observed lesions may not be necessarily related to Lassa infection. Notable was the absence of specific pathological changes in the brain. Some kidneys showed a realignment of cortical tubules thinly lined with squamous epithelium. In one case, tubular cell necrosis occurred in a focus of lymphoreticular infiltrate that contained both small lymphocytes and larger mononuclear cells suggestive of transformed lymphocytes. Elevated levels of blood urea nitrogen and protein-

uria are consistent features of clinical Lassa infection (Monath et al., 1973). The presence of tubular syncytical cells indicates that these symptoms may be a result of a direct virus-induced nephropathy rather than renal hypoperfusion.

Consistently high levels of virus may be found in the blood of acutely ill Lassa fever patients. Walker et al. (1982a) reported 5 to 8 logs of infectious virus in 20 cases hospitalized in Sierra Leone. Vrius was also recovered from liver, spleen, lung, kidney and adrenal glands, the titres were more variable although virus isolation from contaminating blood rather than the tissues *per se* cannot be excluded. The titres recovered from tissue homogenates were rarely higher than those found in the blood of the same patient, although in one patient, nearly 3 logs greater titre of virus was found in infected lung, liver and spleen. It should be noted that in patients who recover excretion of the virus in the urine may persist after the clearance of viraemia (Leifer et al., 1970; Monath et al., 1973; Emond et al., 1982). In one case, virus was detected in the urine up to 67 days from the onset of illness; this patient, hospitalized in London, was not discharged from strict isolation until her urine proved negative for virus on four successive occasions.

The haematological findings in Lassa patients are characterized by a leucopenia (less than 4 million white cells per millilitre) early in infection together with a marked drop in the platelet count and a reduction in prothrombin times (White, 1972). Activated lymphocytes or pseudo-Pelger forms of neutrophils are consistently found in the acute stage of Lassa fever, although this is by no means specific for this disease, being frequently found in other viral haemorrhagic fevers (Knobloch et al., 1980). The finding of platelet numbers not reduced below the level of 50 000 per mm^3 seems to rule out the possibility that haemorrhage in such cases occurs by thrombocytopenia alone. Knobloch and colleagues point out that the presence of intestinal haemorrhage and haemorrhagic conjunctivitis in the absence of stain purpura would indicate blood vessel damage rather than platelet dysfunction. In this context, it is interesting to note the findings of Andrewes et al. (1978) who found that Junin virus was able to replicate in endothelial cells derived from rabbit capillary tissue. Platelets may adhere to virus-damaged cells thereby exacerbating local injury. Indirect damage to capillary endothelium by the deposition of immune complexes may also occur, although there is little evidence yet to show circulating complexes in patients acutely ill with any of the human arenavirus haemorrhagic fevers. Fisher-Hoch (1983), reviewing the problem of understanding haemorrhage that may accompany virus infection, has pointed out that the reported absence of fibrinogen degrad-

ation products or histological evidence of fibrin accumulation in sinusoids or arterioles does not indicate disseminated intravascular coagulation as the primary haemostatic deficiency during acute haemorrhagic infection.

4.3. Argentinian haemorrhagic fever

4.3.1. Epidemiology

This disease has been variously referred to as O'Higgins disease, corn-pickers disease or endoepidemic haemorrhagic fever. The first report was by Arribalzaga (1955) who described the clinical features of an epidemic centred around the provincial town of Junin, approximately 125 miles west of the capital, Buenos Aires. The aetiological agent was subsequently isolated in 1958 by Parodi and colleagues and named after this town. The surrounding region represents the rich, humid farming land known as the Pampas. Maize has been the principal crop, although extensive areas are now being replaced with soyabean. In the late 1950s the endemic area was estimated at just over 6000 square miles, but this has progressively extended both northwards and eastwards to cover a region in excess of 40 000 square miles thereby placing a population of one million or more at risk (Maiztegui, 1975; Fig. 4.3). This 5-fold increase in the size of the endemic area is cause for concern as approximately half of the total Argentinian population of 25 million live within 50 miles of this region.

A study of approximately 16 000 cases between 1958 and 1974 showed the marked seasonal variation in the occurrence of the disease, with the peak in the number of cases identified as coinciding with the harvesting of the maize crop between April and June of each year. Although now largely mechanized, the harvesting of maize during this period was mainly by hand using casual labour who moved into the region solely for this purpose, the majority living in temporary accommodation adjacent to the fields being worked. This seasonal prevalence of Argentinian haemorrhagic fever is explained by the rapid increase in the size of the rodent population, particularly the field voles *Calomys musculinus* and *Calomys laucha* (see Chapter 2). Presumably the virus is spread primarily by rodent contamination of soil vegetation and foodstuffs; although opportunities must exist for the introduction of virus through minor cuts and abrasions, aerosol transmission cannot be excluded and may be of growing importance given the chances of airborne contamination that may result from the use of mechanical harvesting equipment.

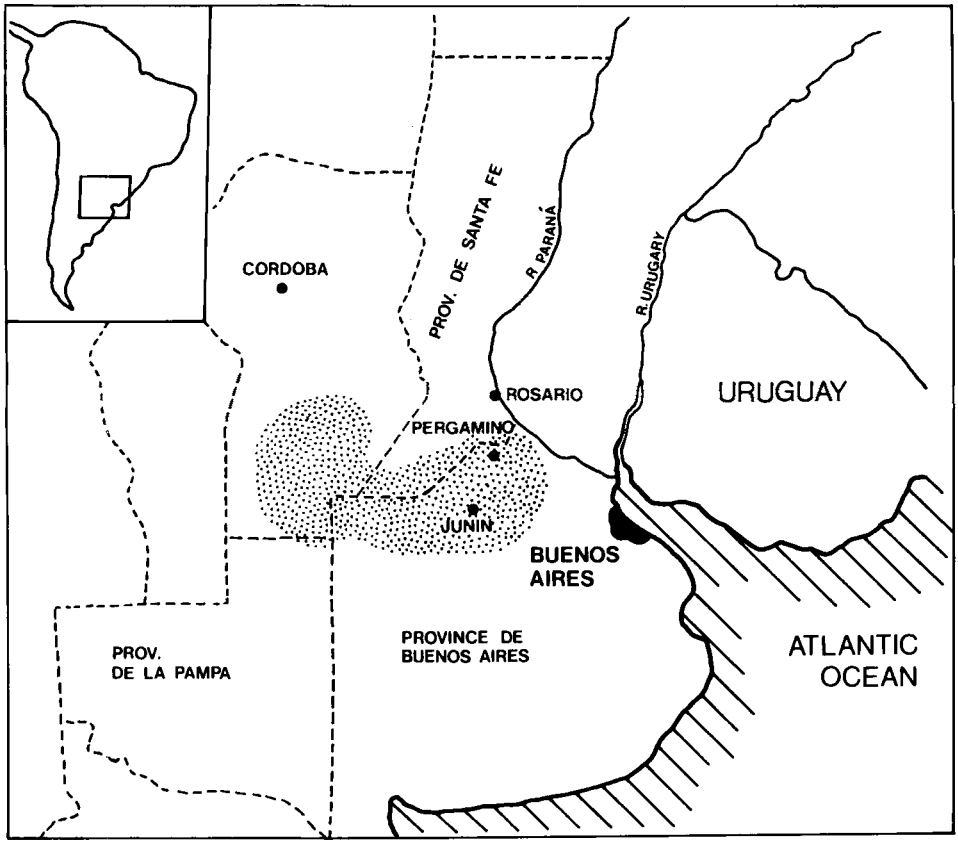

Fig. 4.3. Approximate endemic zone of Argentinian haemorrhagic fever as of 1977 (shown as cross-hatched area) (adapted from Maiztegui, 1975; Maiztegui and Sabattini, 1977).

As expected from the above observations, Argentinian haemorrhagic fever is four times as prevalent in males as in females and largely confined to rural agricultural workers. However, this does not exclude the occurrence of 'urban' cases. An additional factor worthy of emphasis is the report of the simultaneous presence of LCM virus in the rodent population. Evidence of LCM in both humans and rodents in the endemic area was initially obtained by the finding of specific LCM antibody in the peridomestic mouse *Mus musculus* captured in the vicinity of Pergamino, the present epicentre of the Argentine haemorrhagic fever endemic region (Maiztegui et al., 1972). In addition, LCM virus has been directly isolated from *M. musculus* trapped at the same place and time from

which Junin virus had been present in *C. musculinus* (Sabattini et al., 1974).

LCM virus has been directly isolated from the blood of a patient during the acute phase of haemorrhagic illness and Maiztegui (1975) has shown the simultaneous presence of LCM antibodies in approximately 3% of clinical cases suspected of being due to Junin virus infection. However, the majority of these seem to represent cases of prior exposure to LCM virus, but importantly show the theoretical possibility of dual infection of rodents. Owing to the segmented nature of the arenavirus genome, this might in turn result in the emergence of a virus with new epidemiological and pathological properties resulting from genetic reassortment.

4.3.2. Clinical and pathological features

The symptoms of Argentinian haemorrhagic fever are manivested 8–14 days after exposure to Junin virus (Coto, 1974). The primary clinical features of this disease include malaise, fever and haemorrhagic diathesis which includes leucopenia and a marked thrombocytopenia. In addition, there is frequent involvement of the central nervous system. In mild cases, this consists essentially of tongue tremor and in these individuals fever is not prolonged beyond the first week of illness. A transient proteinuria is also present in these mild cases. More severe illness includes a prolonged febrile state accompanied by extensive proteinuria (0.5–1.5 g/litre) and more extensive involvement of the central nervous system, including hyporeflexia or arreflexia, mental confusion, drowsiness and a discrete ataxia. In severe cases, there is muscular hypotonia, arreflexia, ataxia together with convulsions and coma. In the absence of treatment approximately 10–15% of cases are fatal with a terminal shock syndrome. Although increases in white cell and platelet numbers are indicative of a good prognosis, the extent of leucopenia and thrombocytopenia are not considered reliable markers as to the severity of the underlying diseases.

The appearance of complement-fixing antibodies at the onset of clinical recovery suggests that antigen-antibody complexes may play a role in the acute stage of human Junin virus infection, especially as this response is detected immediately after clearance of viral antigens. A study by de Bracco and colleagues (1978) clearly showed, however, that Clq-binding complexes were rarely present during the acute phase, sometimes not appearing until the patient enters convalescence. Other studies have clearly shown the absence of bound immunoglobulin in the glomerulus epithelium.

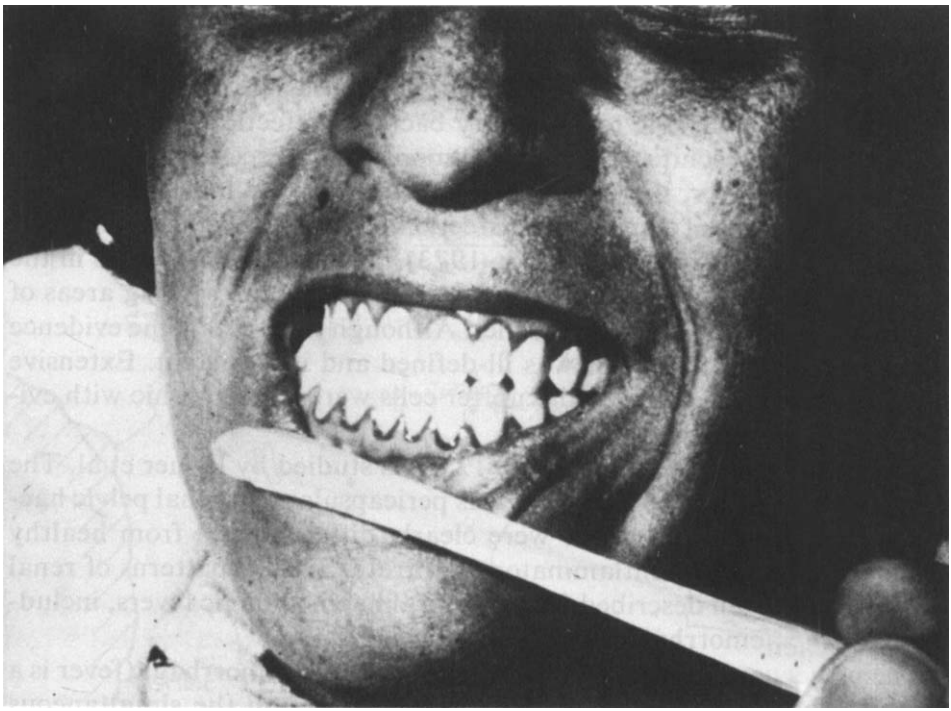

Fig. 4.4. Bleeding from the gums in a case of Argentine haemorrhagic fever (photograph by courtesy of Dr. J.I. Maiztegui).

During the early stages of acute infection, de Bracco et al. (1978) have described a reduction in serum-complement activity in the more severe forms of Argentine haemorrhagic fever. This was accompanied by a reduction in the amounts of C2, C3 and C5, and the appearance of C3 and factor B. The enhanced levels of the latter components may indicate failure to degrade these products early during the acute phase. Changes also occur in the level of blood clotting factors. The bleeding time and clot retraction time becomes prolonged as a result of the thrombocytopenia and the prothrombin time is abnormal in more than 50% of patients. Schwarz (1972) has directly demonstrated a reduction in levels of factor II, VII and X. Some reduction in the levels of factor V and VIII was also seen in the more severe cases where there were signs of intravascular coagulation.

The pathology of Argentine haemorrhagic fever is characterized by general haemorrhage, myocarditis, renal damage and focal necrosis in the liver (Fig. 4.4). As in other human viral haemorrhagic fevers, reticu-

lum cell hyperplasia and erythrophagocytosis in the lymph nodes and erythroid hypoplasia of the bone marrow are found. Elsner et al. (1973) described in some detail the findings at autopsy on a series of fatal cases. Non-specific lesions due to secondary bacterial infection were common and included bronchitis, bronchopneumonia and abscesses in the lungs and heart. However, there is considerable evidence to indicate the non-cytopathic nature of Junin virus infection seen at the light microscopy level (Gallardo, 1970; Elsner et al., 1973). The acidophilic bodies in the liver closely resemble those found in yellow fever, representing areas of degenerated cytoplasmic membranes. Although there was some evidence of nuclear involvement, this was ill-defined and inconsistent. Extensive focal necrosis was evident and Kupffer cells were hypertrophic with evidence of erythrophagocytosis.

The kidneys were enlarged in all 12 cases studied by Elsner et al. The medulla were congested and there was pericapsular and renal pelvic haemorrhages. Areas of necrosis were clearly differentiated from healthy tissue by an extensive inflammatory infiltrate. Similar patterns of renal necrosis have been described for other viral haemorrhagic fevers, including Korean haemorrhagic fever.

The presence of vascular damage in Argentine haemorrhagic fever is a major pathological feature of this disease. Although the simultaneous thrombocytopenia and increased coagulation time are indicative of disseminated intravascular coagulation, there is little pathological evidence to support this as a major disease mechanism (Gallardo, 1970; Elsner et al., 1973).

4.4. Bolivian haemorrhagic fever

4.4.1. Epidemiology

In 1959, an outbreak of haemorrhagic fever was reported to the Bolivian Ministry of Health from local doctors practising in the north-eastern province of Itenez which is adjacent to the border with Brazil (Fig. 4.5). Chinel (1978) describes the area where the epidemics occurred as a high altitude, flat plain known as the 'Llanos de Mojos', an extensive pampas-like region to the east of the Andes chain. Areas of high ground covered by dense forest or bush remain dry during the rainy season when the surrounding areas may become flooded, thereby isolating the rising ground and the villages located at their edges. The climate is tropical and the predominant agricultural activities are cattle and growth of cereal

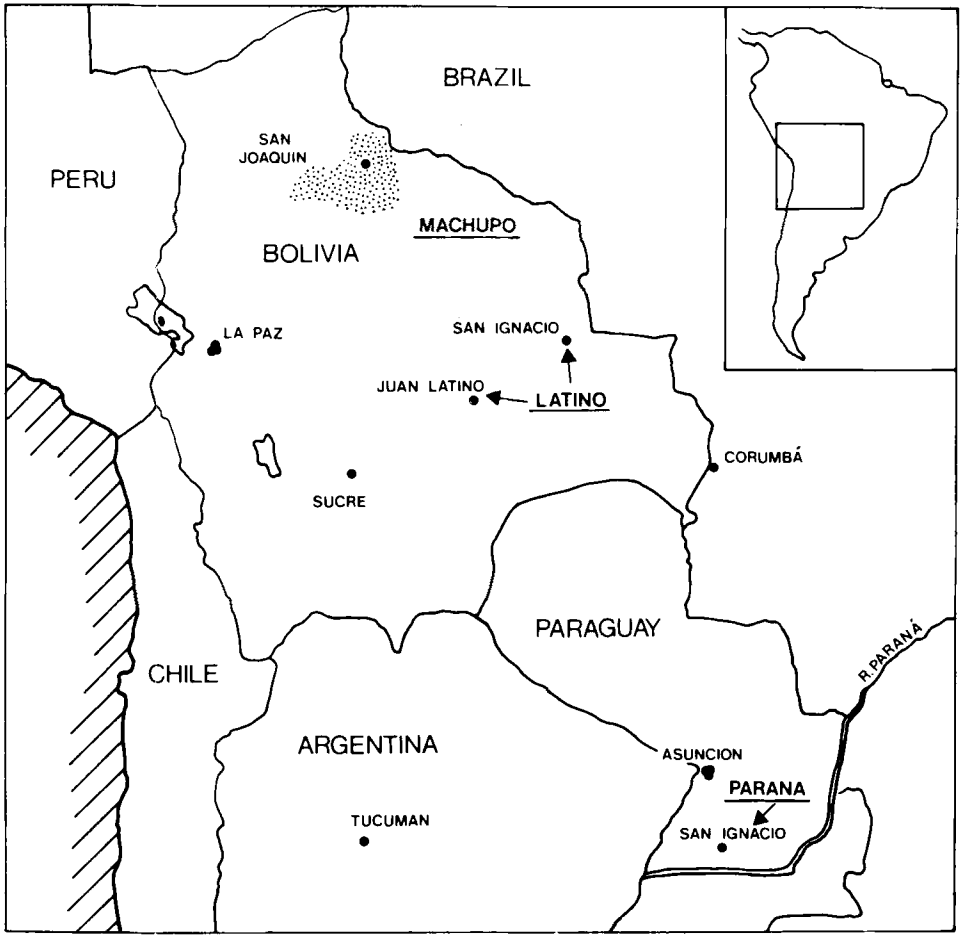

Fig. 4.5. Map of Bolivia and surrounding countries showing the zone of Bolivian haemorrhagic fever and the regions where Latino and Parana viruses were isolated.

crops, rice, yucca and bananas. The cases were largely localized on the so-called 'island' of Orobayaya, and later around the town of San Joaquin some 100 km to the west in the adjacent province of Mamore. Known locally as 'el tifu negro' (black typhus) it was regarded as a possible form of immune typhus until the relationship with the agent of Argentine haemorrhagic fever was established (Mackenzie et al., 1965; Wieberga et al, 1965). At the time of these epidemics, the population of the Itenez province was about 5000 amongst which there were an estimated 470 cases with a 30% mortality. Cases were found chiefly among adult male

agricultural workers, with a marked seasonal occurrence around July and August, the peak of the dry season. As a result of the initial epidemic on the island of Orobayaya, most of the 600 inhabitants fled to the town of Magdalena by the middle of 1962 resulting in a sharp decrease in the number of cases reported from the Itenez province. Indeed, one village in the area was totally abandoned (Mackenzie et al., 1965). Interestingly, no cases were subsequently reported from Magdalena following resettlement.

Although early cases had also been reported from the locality of San Joaquin, these were mainly in individuals living in the surrounding rural areas. However, from late 1962, an increasing number of cases were reported among the residents of the town itself. These patients denied having been beyond the town's perimeter within 2 or 3 weeks prior to the onset of illness. From January to May of 1964, 778 suspected cases of haemorrhagic fever were hospitalized in San Joaquin, of whom 122 died (16%) (Chinel, 1978). During this period detailed epidemiological investigations were begun by members of the Middle America Research Unit of the National Institute of Allergy and Infectious Diseases, then established in Panama. This group successfully isolated the causative agent by inoculating infant hamsters with an extract prepared from the spleen of a 2-year-old boy who had died of Bolivian haemorrhagic fever (Johnson et al., 1965b). This isolate, designated the Carvallo strain, was used to prepare an infected brain suspension as a source of antigen for serological surveys. The name Machupo was taken from a nearby tributary of the Itenez river which passes through the region where the epidemic was centred. By means of a CF test using Machupo virus antigen, Mackenzie (1965) reported a detailed survey of urban Bolivian haemorrhagic fever occurring among hospitalized and non-hospitalized cases. It was estimated that 84% of survivors hospitalized with suspected Bolivian haemorrhagic fever and antibodies to Machupo virus. Although a number of deaths may have erroneously been attributed to virus infection, this was almost certainly an underestimate given the relatively insensitive nature of this serological method (see Chapter 3). Based on these results, the fatality rate was estimated at 19% among serologically confirmed cases. Although very high, some improvement in survival rate as compared to the original estimate of 30% may have been due to more accurate reporting and/or the application of better supportive therapy. Mackenzie and colleagues found repeated clustering of cases by household with over 90% of cases being restricted to the southern half of the town. A programme of rodent eradication begun in May 1964 resulted in a dramatic fall in the number of reported cases, with no further cases being reported

more than 2 weeks after rodent control was extended over the whole quarter square mile township.

The incubation period appears to be up to 16 days after exposure, as estimated from both the observed termination of cases after the introduction of control measures and the development of the disease in a number of individuals entering the town from non-endemic areas (Mackenzie, 1965). There was little evidence of human transmission in San Joaquin, despite the clustering of cases in particular households. The latter occurred sporadically, consistent with the concept that peridomestic yards become infested with rodents carrying Machupo virus. Another factor against person-to-person spread during the epidemic was the absence of nosocomial transmission to health-care staff attending the hospitalized cases. The exceptions were two cases that were reported among the wives who visited their husbands in Panama after the latter had been evacuated with acute Bolivian haemorrhagic fever from the endemic region. During the course of the epidemiological investigation in Bolivia, little evidence was obtained to suggest asymptomatic infections were occurring although again the limited sensitivity of the tests used at the time may not have detected low level antibody responses.

Kuns (1965) has described the application of the rodent eradication programme in restricting the spread of Machupo virus via its natural host, *Calomys callosus*. This is a unique situation in the control of human arenavirus disease, possibly owing to the ecology of the rodent host. The use of ordinary mouse traps proved effective in reducing the numbers of peridomestic rodents, over 96% of caught animals being *C. callosus* species.

4.4.2. Clinical and pathological features

As with cases of Argentine haemorrhagic fever (see Section 4.3.2) onset of symptoms is insidious, the disease symptoms developing slowly over a 2 to 4-day period within 2 weeks of exposure to Machupo virus. Tiredness, general malaise and mild to severe muscular aches, particularly in the lower back were common among the four cases reported in detail among the team investigating the San Joaquin epidemic (Stinebaugh et al., 1965). Two of these patients also complained of upper respiratory tract symptoms including a sore throat, a non-productive cough and a stuffy nose. Headache and extreme cutaneous hypersensitivity were also prominent features during the prodromal phase. In most cases of Bolivian haemorrhagic fever, maximum temperature between 38° and 40°C occurred by the third day of illness. The fever typically was unremittent,

accompanied by severe frontal or retro-orbital headaches and excruciating back pain, with severe joint and muscular discomfort. During this period, anorexia or nausea and vomiting together with epigastric pain and diarrhoea were common. The febrile phase lasts at least 5 days during which time patients begin to exhibit haemorrhagic manifestations. These include erythema of the palate, conjunctivitis and the development of petechiae over the upper trunk, face and neck. Bleeding occurs from the gums, nose, stomach and occasionally the uterus, but the loss of blood is not large and not to be regarded as a serious threat to life except in the circumstance of intractable epistaxis (Johnson et al., 1967).

Stinebaugh et al. (1965) distinguished a second, vascular phase which immediately followed the febrile period. This was marked by the development of cardiovascular lability and hypotension. Evidence of capillary leakage was seen, including periorbital oedema and proteinuria. During this phase, the skin appeared clammy and cold to touch with a rapid weak pulse alternating with warm extremities, slow pulse and dry skin. In one of four patients followed by Stinebaugh et al. sustained hypotension led to shock, which subsequently responded to treatment. Involvement of the central nervous system is frequently seen, ranging in severity from tongue tremor to progressive and severe encephalopathy. Other manifestations included irritability, lack of awareness, delirium and hallucinations in the more severe cases.

Serum electrolyte levels appear normal during the acute illness, and in a limited study serum transaminases were within normal limits. In common with Junin infection of man, there appears to be little hepatic involvement during acute Bolivian haemorrhagic fever in contrast to the hepatotropism of Lassa virus (Leifer et al., 1970).

Serum creatinin levels remain normal except in severe cases which also show an increased clearance rate. In the milder cases, other tests for kidney function remain normal, although as already mentioned, proteinuria may accompany extensive cardiovascular leakage.

Leucopenia is observed in most patients. Although the differential white cell count remained normal during the early stages of the illness, relative lymphocytosis was seen as the number of white cells returned to normal later during the acute infection (Stinebaugh et al., 1965). All patients showed a thrombocytopenia, but platelet counts never dropped below 50 000 per mm^3. Bilirubin and prothrombin times remained normal. Stinebaugh and colleagues also pointed out in their paper that supportive therapy similar to that given to patients with Korean haemorrhagic fever was appropriate for the management of patients in the vascular phase of Bolivian haemorrhagic fever. Mild hypotension was treated

by placing patients in the Trendelenburg position and administering either human serum albumin, or vasopressors in the more severe cases.

Patients entering convalescence show evidence of continuing cardiovascular lability and hypotension with a rapid pulse after light exercise. These features subside within 3 weeks after the disappearance of neurological signs and the patient becomes afebrile (Stinebaugh et al., 1965). Some hair loss was also noticed, but regrowth occurred after 3 months when most patients recovering from acute illness regained a sense of well-being. Although Bolivian haemorrhagic fever in man clinically resembles the Argentinian syndrome, hair loss appears to be an additional feature of Machupo infection (Mackenzie et al., 1954).

A limited number of fatal cases have been subjected to thorough pathological investigation. Autopsies were carried out on eight patients by Child et al. (1967), all of whom had presented with the typical clinical features of Bolivian haemorrhagic fever. Gross pathological changes included extensive signs of haemorrhage in the gastrointestinal tract and acute congestion of all organs. Pulmonary haemorrhages were noticeably absent, however, although intracranial haemorrhages occurred in half those patients studied. The most prominent microscopical changes were seen in the liver. In all cases, the number and size of Kupffer cells were increased and there was noticeable eythrophagocytosis together with increased eosinophilic staining of hepatic cells. Lymphocytic infiltration was seen only in the periportal regions with frequent mononuclear cells present in the sinusoids. Focal necrosis was occasionally seen, although the hepatic architecture was preserved. A curious feature was the presence of acidophilic bodies, 10–20 μm in diameter, free in the hepatic sinusoids and sometimes contained within Kupffer cells; these may have resulted from the direct cytopathic action of virus on hepatocytes or have been of platelet origin. A widespread hypoplasia typical of viral haemorrhagic fevers was seen throughout the reticuloendothelial system and particular bodies within macrophages were clearly evident in both spleen and lymph nodes. A number of so-called 'activated' reticulum cells with cytoplasmic inclusion bodies were clearly visible above the background of lymphocytes. A number of early papers compared both the clinical syndrome and pathological features of Bolivian haemorrhagic fever with cases of Korean and other Asian fevers, the latter clearly showing renal involvement. Although hyaline cases were seen in the kidney tubules, Child et al. could not find any major pathological changes in the kidneys of the patients who died of Machupo infection.

CHAPTER 5

Arenavirus infections of animals

5.1. Natural and experimental infections of rodents

5.1.1. Lymphocytic choriomeningitis virus

The symptoms accompanying acute LCM virus infection in mice have been described in detail by Hotchin (1962). Symptoms of acute infection become apparent 5 days after intracerebral infection, commencing with ruffling of the fur, a hunched posture and facial oedema. The signs increase rapidly in severity accompanied by weight loss, immobility and hypersensitivity to external stimuli. Convulsions and paralysis of the limbs precede death by day 8 after infection. Hotchin describes the animals on death as assuming a characteristic posture of spinal flexure, with extension of the neck and hind limbs. Although this appearance is typical of LCM virus, the author draws attention to the similarity with some strains of mouse hepatitis virus and anaphylactic shock. An increase in the virus dose may cause earlier onset of disease, although high doses result in a decrease in both severity and mortality (see Chapter 9). Although this profile of so-called 'classical' acute LCM disease is usually obtained by intracerebral infection of adults, certain viscerotropic strains exist with the capacity to induce severe lethal disease by the intraperitoneal route. Subcutaneous or intranasal inoculation of adults causes only slight disease with the animals recovering to develop complete immunity.

A full description of these varying disease profiles can be found in the early reports of Traub (1936a, b, 1939) and have been reviewed extensively by Lehmann-Grube (1971).

Although there is some variation between strains of LCM virus, acute disease is generally accompanied by a significant myelitis and a generalized lymphocytic infiltration of the major organs. The latter results in enlargement of the liver and severe hepatitis leading to necrotic foci of the liver parenchyma. These cells become eosiophilic with the development of cytoplasmic vacuolation and develop in areas of adjacent sinusoids (Tosolini, 1970). However, there is no evidence of reduced plasma levels of liver-produced transaminases or other serum enzymes throughout acute LCM infection (Mahy et al., 1964). Splenic and lymph node hyperplasia is readily apparent and there is both a pneumonitis and cardiac involvement. Perhaps the most detailed account of LCM pathology in the mouse is provided by the early report of Lillie and Armstrong (1945). In a study of some 760 specimens of infected brain, the absence of lesions in the neuronal parenchyma was particularly noticeable. Meningeal infiltration reached a peak between 6 and 9 days after infection and was found to be greatest on the base and in major fissures, particularly in the region between the brain stem and overlying cortex. Infiltration of the choroid plexus was greatest in the 3rd and 4th ventricles, even in mice inoculated by non-intracranial routes. Findlay and Stern (1936) independently confirmed that nerve cells even in close proximity to infected membranes remained remarkably free of pathology. Intraventricular exudates composed largely of lymphocytes were common, although apparently related to the introduction of foreign matter by the intracerebral route rather than virus infection *per se*, as exudates were rarely seen in animals infected by other routes. Inflammation of the meninges and choroid plexus was found to be distinctly more focal in nature when animals received virus via the intraperitoneal, intranasal, subcutaneous or intravenous routes. Lymphocytic infiltration of the spinal meninges in mice has generally been found to be less extensive (Traub, 1936b; Findlay and Stern, 1936; Rivers and Scott, 1936a).

LCM virus will multiply in many visceral organs of the adult mouse, and this has been well documented (see Lehmann-Grube et al., 1971 for review of early data). After intracerebral inoculation, the virus replicates in the neural membranes regardless of the strain. However, many laboratory strains of LCM virus will grow to higher titres in liver and spleen although this may be dependent on the previous passage history of the

virus stock used; virus previously grown in guinea pigs spread to grow more readily to visceral organs when subsequently injected intracerebrally into mice (Schwartzman, 1946). After intracerebral inoculation the first signs of histological abnormalities are seen in the lymph nodes 3–5 days after inoculation consisting predominantly of small lymphocyte destruction which progresses from the cortex to involve the whole node by day 7 (Hanaoka et al., 1969). At the same time, extensive proliferation of both reticulum and basophilic lymphocytes occur at the marginal cortex. Similar changes are found in the white pulp of the spleen and in the thymus which rapidly atrophies as the disease progresses.

Some strain differences in tissue tropism can be seen also by intraperitoneal inoculation. Lehmann-Grube (1964) found that the WE strain grew to high titres in both the brain and viscera of animals peritoneally infected. In contrast, the E-350 strain, derived from the Armstrong CA 1371 isolate, grew poorly in the livers and kidneys of animals infected by the same route (Lehmann-Grube, 1971). Although LCM strains are often regarded as being either viscerotropic or neurotropic, the disease patterns seen during acute LCM virus infection is often related to route of inoculation and the degree of subsequent viraemia allowing spread of virus to other target organs. For example, after intracerebral inoculation of 6-week-old SWR/J mice with the Armstrong CA-1371 strain, virus titres in the serum exceed 150 LD_{50} per millilitre 4 days after infection (Buchmeier et al., 1980a) thus allowing ample opportunity for virus dissemination. However, there does not seem to be a simple correlation between viraemia levels and the numbers of virus-infected cells in the bone marrow (Bro-Jorgensen and Volkert, 1972a). It should be noted here that the level of virus in the blood during acute infection is generally 1–2 logs lower than that seen in persistently infected animals. The titre of infectious virus in various tissues also is greatest 4–5 days after infection, and in general terms may exceed 5 $\log_{10}$ pfu in extracts of whole spleen. Detailed information on the growth of LCM virus in various organs including liver, spleen, lungs, kidneys, thymus and brain is comprehensively reviewed by Rowe (1954), Lehmann-Grube (1971) and Hotchin (1971a). It has been recognized that the extent of neurotropism or viscerotropism seen on comparison of different LCM virus strains may in part be due to the passage history of virus stocks resulting in the preferential selection of either of the two variants. For example, Hotchin et al. (1971) described a mixture of two different plaque types in mouse-passaged virus. Clear plaques were predominant in mouse brain suspensions titrated on BHK-21 cell monolayers whereas virus grown by sequential passage in mouse liver gave turbid plaques which could interfere with the

cytopathic effect of the clear variant. Intracerebral inoculation of adult mice with either variant produced a lethal infection, although survival was prolonged in the case of the turbid plaque variant. A more marked contrast was seen in newborn mice. All animals infected with the clear plaque variant succumbed to an acute fatal infection whereas the turbid plaque variant induced a chronic persistent infection. These results are compatible with the development of interfering particles by growth in selected cell types which prevent acute lethal disease (see Chapter 8).

Viral antigen may readily be detected in the brain 24 h after infection by the intracerebral and intravenous routes, somewhat later if the virus had been given by other routes. Significantly, antigen is not found by immunofluorescence in the adjacent parenchyma, confirming the pathological data which shows that LCM virus infection is restricted to the neural membranes.

An important extension to these early findings is the recent description by Oldstone et al. (1984) of LCM viral antigens in the pituitary of infected mice. Although the tissue appeared histologically normal, serum growth hormone levels were reduced in chronically infected animals, the degree of which correlated with virus replication specifically in cells of the anterior lobe producing the hormone. This reduction appears specific as prolactin levels remained normal and there appeared to be no significant infection of pituitary cells specializing in prolactin synthesis. As persistently infected mice show stunted growth, a direct relationship exists with LCM persistence and a marked reduction in glucose metabolism. This was confirmed by passive restoration of normal glucose levels on transplantation of cells secreting growth hormone. These results confirm earlier *in vitro* studies which showed that persistent LCM virus infection of neuroblastoma cells resulted in a drop in enzyme levels involved in acetylcholine metabolism (Oldstone et al., 1977). Other non-differentiated cellular functions vital for cell survival remain unaffected. Thus, chronic LCM infection is accompanied by an apparent discrimination at either the transcriptional or translational level in its effect on normal host cell function.

After intraperitoneal LCM virus infection, antigen appears 3 days later in the liver parenchyma, lungs and the red pulp of the spleen. These studies were developed further by Tosolini (1970) who followed the course of antigen expressed in outbred mice infected intravenously with the WE strain of LCM virus. The pathogenesis of the infection in the liver was particularly interesting. Viral antigen was detected in approximately 30% of cells by day 3. Small foci of infected hepatocytes were apparent at 24 h, but infection of the liver parenchyma did not develop extensively until day 4 when over half of all Kupffer cells seen had detectable anti-

gen. Immunoglobulin of the IgG class was deposited on the periphery of the necrotic foci, often in the cytoplasm of otherwise histologically normal hepatic and mononuclear cells. In the same study viral antigen was apparent in the walls of blood vessels by day 4 and became rapidly widespread throughout the meninges and epithelial cells of both the choroid plexus and the ependymis. Interestingly, small numbers of infected large mononuclear cells were present in the subarachnoid space 18 h after inoculation; similar cells with a bilobed nucleus and an extensively vacuolated cytoplasm were also seen in the blood. These were positive for viral antigen in both the cytoplasm and nucleus.

The results of Tosolini (1970) are consistent with the theory that hepatitis involves prior infection of Kupffer cells, leading to a delay of some 12 h prior to transmission to hepatocytes, a similar mechanism that has been suggested for ectromelia virus (Mims, 1959). Up to 95% of unstimulated peritoneal macrophages may support viral antigen production (Mims and Subrahmanyan, 1966) although there appears to be some marked variation between host and virus strains (Tosolini and Mims, 1971). Similar evidence that LCM virus infects peritoneal mouse macrophages was obtained by Schwartz et al. (1978). Both the WE and E-350 strains multiplied in these cells without affecting normal macrophage functions such as phagocytosis and enzyme production. However, the total number of infectivity units produced by a single cell is much lower in comparison to the titre obtained with mouse fibroblast cells.

Working with the WE strain, Lohler and Lehmann-Grube have documented the immunopathological changes in LCM-infected NMRI mice. After intraperitoneal infection, virus was detected in the spleen and thymus 24 h later (Lohler and Lehmann-Grube, 1981). Two days after infection, viral antigen was found in small foci of splenic macrophages in the marginal zone spreading as the disease progressed to become confluent and to involve first the white, and then the red pulp. This reached a maximum 5 days after infection and thereafter decreased. Similarly, viral antigen was detected by immunofluorescence in the lymph nodes first in the macrophages and reticular cells of the marginal sinuses, thereafterwards spreading rapidly to the cortices via the intermediate sinuses. In many respects, the histological changes in the spleen resembled those described by Murphy et al. (1977), with extensive necrosis of both the white and red pulp of the spleen. However, a unique observation was that a large proportion of blast cells were of B-cell origin as revealed by the detection of surface immunoglobulin. Both blast cell proliferation and extent of necrosis peaked at day 5, corresponding to the maximum accumulation of viral antigen. Similar alterations were seen in the lymph

nodes, although necrosis was not so severe. Initial lesions in the internodular cortex spread towards the thymus-dependent area of the deep cortex, with many lymphoblasts being positive for surface immunoglobulin as seen in the spleen. Changes in the thymus also followed a similar pattern, with first evidence of infection being an activation of macrophages followed by extensive necrosis of cortical lymphocytes.

The recent development of cDNA probes representing viral gene sequences has opened up an entirely new approach to understanding LCM virus pathogenesis. Hybridization of virus-specific sequences has been achieved *in situ* by Southern et al. (1984) using whole body sections of persistently infected mice to reveal the distribution of the LCM viral genome. Interestingly, genetic material was found in several previously unexpected tissues, including the stomach mucosa and the ductus deferens. Although these studies were carried out using mice infected at birth, this approach should have many applications in the study of acute and chronic arenavirus infections. For example, the studies by Southern and colleagues showed a discrepancy between levels of viral genome in infected tissues and titres of circulating virus; animals infected at birth showed comparatively low levels of genome activity 5 days later when the titre of virus was high and conversely titres were low by 180 days after a substantial increase in viral nucleic acid levels. Two other observations are worthy of note from this study. Firstly, there were significant and specific differences in the levels of LCM genome sequences in the hepatic parenchyma between persistently infected mice of different haplotypes. Secondly, embryos within carrier mothers differed in the extent of virus activity. These findings demonstrate the potential usefulness of extending these studies for comparative work on other viruses, particularly Junin and Lassa fever, where useful information may be obtained at the tissue level as to differences between virus isolates and how these viruses are maintained in the natural rodent population.

Several important studies have demonstrated that acute LCM virus infection in mice leads to a very marked reduction in haemopoiesis. Rowe (1956) showed that X-irradiation of adult mice conferred some protection from LCM; this protective effect increases up to a dose of 500 rads (Hotchin and Cinits, 1958; Hotchin and Weigand, 1961a) a dosage level which normally depletes the circulating lymphocyte population but repopulation of the spleen by haemopoietic stem cells from bone marrow may follow. Above this level repopulation is inhibited and transfer of bone marrow cells from syngeneic normal animals is required to prevent death from the irradiation. Although infected adult mice showed no signs of disease if given a sublethal dose of X-rays 24 h prior to inoculation, the

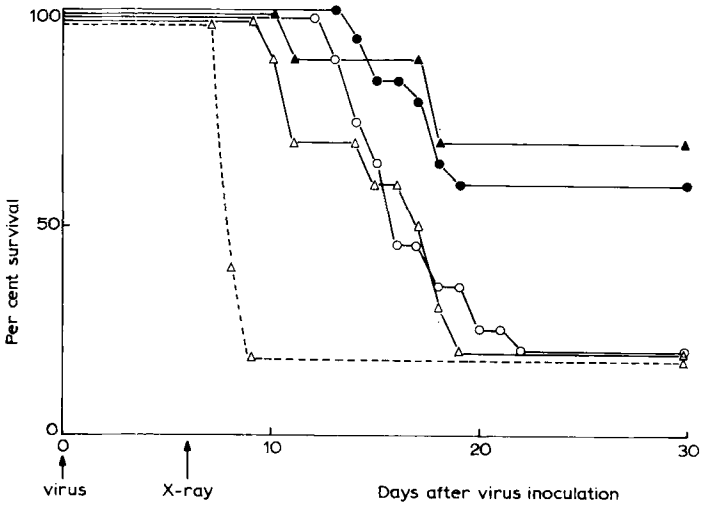

Fig. 5.1. The effect of reconstitution with foetal liver cells on the mortality of irradiated mice acutely infected with LCM virus. Animals were exposed to X-rays 6 days after infection and liver cells were injected within 3 h. ----, Survival curves for mice infected either by the i.c. (△) or i.p. (○) routes. ——, The survival curves of the two groups after reconstitution (from Bro-Jorgensen and Volkert, 1972a).

subsequent titres of virus in both blood and the brain developed to the same levels as seen in the control, non-irradiated animals. All histological evidence of LCM virus disease was absent in mice depleted by X-ray treatment, there being no evidence of lymphocytic infiltration in tissues or hepatocellular necrosis (Collins et al., 1961); the severe experimentally induced leucopenia lasted about 10 days, approximately the length of time of protection conferred by X-ray treatment. Bro-Jorgensen and colleagues have observed that persistently infected mice showed a marked sensitivity to X-irradiation, the lethal dose of X-rays being much lower compared to normal controls. In contrast to the protection conferred by X-ray treatment prior to inoculation, delay of irradiation until 6 days after infection had no effect on the high mortality rate, although the mean survival time was prolonged from 8 to 16 days (Bro-Jorgensen and Volkert, 1972a). However, the transplantation of foetal liver cells 3 h after X-ray treatment did produce a significant 3 to 4-fold enhancement in the survival rate of acutely infected animals (Fig. 5.1). Again as in the earlier work of Hotchin and colleagues, there was no evidence of these effects being directly correlated with changes in virus titres, and the observed haemopoietic regeneration by foetal liver cells in the continuing presence of circulating virus indicates that LCM virus does not have a

direct damaging effect on haemopoietic cells, although clearly the effect is virus-induced. This is further evidenced by the development of unique symptoms in irradiated mice which include severe anaemia and widespread haemorrhages in many tissues in the absence of cerebral lesions.

Histologically, the bone marrow of irradiated, acutely infected adult mice show depletion of both mature erythroid and granuloid cells, although the proportion of blast cells is increased (Bro-Jorgensen and Volkert, 1972a). Such haemopoietic dysfunction can be demonstrated by the complete abolition of stem cell colony formation *in vitro* cultured in the presence of LCM virus (Bro-Jorgensen and Volkert, 1972b).

The pathology of LCM virus in hamsters has received some attention since there have been a number of human cases resulting from the handling of animals persistently infected with the virus, either in laboratory colonies (Baum et al., 1966; Armstrong et al., 1969) or as pets (Biggar et al., 1975; Deibel et al., 1975). Smadel and Wall (1942) found that adult Golden Syrian hamsters can be readily infected by either intracerebral or intraperitoneal routes, producing a systemic disease involving all organs from 5 days after infection. Particularly high levels of virus were recovered from the brain and spleen of infected animals although there were few deaths. Virus was excreted both in the urine and faeces. Only three of 54 animals inoculated with LCM virus became ill 3–4 weeks after infection; clinical signs included loss of weight, dehydration, occasional tremors and prostration. Only minor histological abnormalities were noted, primarily in the spleen.

Newborn hamsters are also susceptible to LCM virus. Volkert and Larsen (1965a) found that two-thirds of animals infected intraperitoneally died within 2 weeks. Antibody conversion was complete in the survivors by the age of 6 months having cleared the virus from the blood. Parker and colleagues (1976) re-examined in detail the response to LCM virus in newborn hamsters, with slightly different results.

Animals infected within 24 h after birth develop a chronic viraemia and viruria with approximately half of the infected hamsters developing vasculitis and glomerulonephritis and die at about one year of age. The remaining animals cleared the viraemia by about 6 months, although shedding of the virus in the urine may continue into the second year of life. In diseased animals infected as newborns, signs of illness developed from 2 months of age and consisted of hunched posture, loss of fur quality and weight, and blepharitis. High titres of virus were found in the kidney although all organs examined contained significant levels of virus including the liver and lungs. The lowest amount of virus were found in the

blood and brain. This contrasted with healthy animals which showed negligible titres of tissue-associated infectivity, although virus was readily demonstrated in the kidney. Histological examination of diseased animals from 12 weeks after infection showed extensive lymphocytic infiltration in many organs, including the liver, lungs, spleen, brain and kidneys, with severe periportal inflammation present in the liver. In the later stages of the disease, pathological changes in the kidney became pronounced with glomerular basement thickening with occasional lesions in the glomerulus resembling those seen in lupus erythematosis. Renal arteritis accompanied the glomerular changes and immune complexes containing viral antigen were detected in both the arteriole walls and the basement membranes.

In contrast, adult hamsters did not develop disease when exposed to LCM virus; the viraemia was of relatively short duration and had ended 60 days after inoculation. However, as with survivors among newborns infected at birth, virus continued to be shed via the urine for periods exceeding 6 months. Taken together, these findings illustrate the tropism for the kidneys or possibly the urogenital tract in hamsters and even a symptomatic infection may lead to prolonged shedding of infectious virus into cage litter. Furthermore, mice born to persistently infected mothers may themselves become virus carriers. A proportion developed the chronic progressive disease seen in experimentally infected newborn hamsters, whereas the remainder continued to harbour the virus eventually transmitting the virus to the next generation.

Guinea pigs are susceptible only to certain strains of LCM virus. The WE strain, originally isolated from human tissue by Rivers and Scott (1935), is highly lethal for this rodent, even if applied to unscarified skin (Shaughnessy and Zichis, 1940; Smadel and Wall, 1940). Animals die within 2 weeks following a progressive febrile illness with anorexia, loss of hair quality and loss of weight. The animals become prostrated and in the later stages salivation and respiratory difficulties may develop. The WE strain is infectious regardless of route of inoculation and as little as one infectious unit is sufficient to induce a lethal infection. (Lehmann-Grube, 1971; Buchmeier and Dutko, 1980). Replication appears to occur in all organs with particularly high levels of virus being found in the spleen, lymph nodes and lungs (Berda et al., 1964) and urine for up to 6 weeks, often after the virus has been cleared from the circulation (Traub, 1936b). Many other strains of LCM virus do not cause overt disease in guinea pigs although virus can be recovered from many organs including the spleen and lungs, albeit at much lower titres. For example, animals are resistant to as much as 10^6 infectious units of either CA-1371 (Arm-

strong) or E-350 strains. These differences in biological properties reflect differences in the RNA genomes of these strains (Dutko and Oldstone, 1983) although it is still unclear as to how such variations are manifested *in vivo.*

5.1.2. Lassa virus

Several studies have attempted to investigate the pathology of Lassa virus infection in its natural host, *Mastomys natalensis* (Monath et al., 1974a; Wulff et al., 1975; Walker et al., 1975). Both adult and neonatal animals were infected intraperitoneally by Walker and colleagues. Infection of animals at any age resulted in an asymptomatic infection. Virus was recovered from various organs of animals infected at birth for as long as 74 days after infection with particularly high titres being found in the spleen, liver and lung. High levels of virus in the kidneys and urine was also a consistent finding. Although no significant histopathological changes were found in neonatally infected animals, immunofluorescent analysis revealed the presence of viral antigen in the liver, megakaryocytes and the thymus. Foci of infection were also seen in the kidney, brain and bladder epithelium. Similar findings of virus in extracts of major organs were also seen in infected adults, although a tropism for the spleen and lymph nodes was particularly prominent. Histological changes were confined to the brain with signs of meningoencephalitis. One interesting observation was the persistence of virus in some infected adults as a result of continuing virus replication in certain organs such as the lymph nodes and brain. Continual shedding of virus in the urine in persistently infected *Mastomys* rodents is believed to be the major source of viral contamination into the environment and the establishment of virus persistence in newborn animals is a major factor in the maintenance of a reservoir of infection in the rodent population (see Chapter 2).

Outbred newborn white mice are susceptible to intracranial inoculation of Lassa virus (Buckley and Casals, 1970). Little has been published as to the pathology of Lassa infection of laboratory mice. Among a litter of 20 neonates infected by Buckley and Casals with a human acute serum sample, all but one survived the infection, with virus continuing to be excreted in the urine for nearly 2 months after infection, despite the development of a viral antibody response. However, over half of adult animals inoculated with Lassa virus after passage in cell culture succumbed to the virus within one week of infection. Moribund animals showed convulsions, hind-limb paralysis and respiratory difficulties. Although no histological examination was reported, these signs indicate a

choriomeningitis similar to that seen in adults infected with LCM virus.

The guinea pig has been shown to be a useful laboratory animal model for the study of Lassa virus infection (Walker et al., 1975; Jahrling et al., 1982). Walker et al. (1975) found that intraperitoneal inoculation for the purpose of raising specific antisera resulted in death in two-thirds of adult animals so infected. The outcome of infection in guinea pigs is dependent on the strain of animals used: strain 13 and strain 2 animals are uniformly killed by doses as low as 2 pfu per animal (Jahrling et al., 1982). In the latter study infected outbred (Hartley strain) animals survived, many having undergone inapparent infections as assessed by seroconversion and resistance to challenge with homologous virus. The infection in strain 13 animals was characterized by a rapid elevation in viraemia by day 10 with a corresponding antibody response similar to that seen in surviving animals. However, the viraemia levels in the latter group were almost one log lower, suggesting that survival may in part be due to a restriction on the extent of virus replication.

Virus replication occurs in many organs, including the liver, spleen, lungs, kidneys and the lymph nodes. Jahrling et al. (1982) reported the absence of virus in the brain, although infectious virus was found in this organ in the earlier study of Walker et al. Particularly high levels of virus are consistently found in the lungs and antigen can be readily detected by immunofluorescence. However, the extent of virus activity in any organ is not reflected by the extent of tissue damage, with only a mild to moderate interstitial pneumonia in the lungs, limited foci of necrosis in the spleen and minimal inflammation of the liver in those animals with acute infection. More conspicuous lesions were noted in moribund animals sacrificed by Walker et al. (1975): consolidation in the lungs, pleural infusion and ascites were noted together with microscopical lesions being seen in the lungs, liver and heart. A pulmonary oedema with formation of alveolar hyaline membranes and pleural effusions clearly resembles human infection.

There is a clear difference in the pathology of experimental guinea pig infection and Lassa virus-infected primates and man. Despite the similar tissue tropism in all susceptible host species, the lesions found in the guinea pig are substantially less severe. For example, the guinea pig liver does not appear to be a major site of virus activity whereas in primates this organ frequently contains the greatest amount of infectious virus and hepatocellular necrosis is prominent. By contrast, guinea pigs show a marked myocardiopathy, a feature absent in human Lassa infection, although the latter may reflect inadequate investigation of fatal cases at necropsy as there are electrocardiographic changes during the acute ill-

ness. Although a useful species for the primary isolation of virus from clinical specimens, these differences in pathology make the guinea pig less suitable for detailed study of Lassa virus pathogenesis.

Jahrling et al. (1981) have described the adaptation of Pichinde virus to guinea pigs as an alternative animal model for Lassa virus infection. The virus was sequentially passaged through inbred strain 13 guinea pigs, virus being recovered from the spleen at each successive pass. At the eighth passage the virus was uniformly lethal when inoculated subcutaneously at doses as low as 1 pfu. This contrasted with outbred animals where infections were initiated at similar low dose levels, although mostly inapparent. Whereas the viraemia in outbred animals rarely exceeded 3 logs of virus, the process of adaptation to strain 13 animals produced viraemia levels in excess of 5 logs 16 days after infection despite the onset of a humoral antibody response. Infected inbred animals developed a severe leucopenia immediately prior to death and serum transaminase levels were elevated. This latter indication of hepatic injury was confirmed by the finding of moderate to severe hepatocellular necrosis in the livers of moribund animals although inflammatory infiltration was minimal. Many cells showed the presence of viral antigens, a finding similar to that seen in the liver of both man and rhesus monkeys infected with Lassa (Jahrling et al., 1980; Winn et al., 1975). A further finding in these animals was a high concentration of viral antigen in the adrenal cortex; notably over 8 logs of infectious virus was recovered per gramme of infected tissue in marked contrast to an almost negligible level of virus in animals infected with the original 'parent' virus at the same time after infection. Similar differences were recorded for other organs including the pancreas, again emphasizing that possible disturbances of corticosteroid and other hormone levels may contribute to the fatality of the infection. Involvement of the lungs was apparent as interstitial pneumonia, typically with thickened alveolar septae and associated antigen-positive macrophages. An interesting observation was the presence of virus in the salivary gland parenchyma indicating that virus transmission may occur by aerosols of infected saliva. As the pathology of Pichinde infection more accurately resembles Lassa infection of primates this system offers several advantages. In addition, detection of neutralizing antibody to Pichinde virus presents similar difficulties to detection of neutralizing antibody to Lassa virus (see Chapter 3), a problem that can be more readily examined at the whole animal level using the guinea pig – Pichinde virus pairing.

5.1.3. Machupo and Junin viruses

Johnson et al. (1966) described the condition of naturally infected *Calomys callosus* brought to the San Joaquin laboratory during the epidemic of Bolivian haemorrhagic fever in that area. A few animals presented with intermittent or continuous clonic convulsions, and one showed signs of both external and internal haemorrhage and could not stand. However, there were no gross abnormalities seen on dissection, although Machupo virus was easily recovered from the spleen of these and the majority of other animals with no signs of illness. Virus passaged into hamsters produced neurological symptoms similar to those seen in human infections. All isolates characterized showed a close serological relationship with virus recovered from acutely ill patients and could be passaged readily in hamsters by intracerebral inoculation of infected brain extract.

Machupo virus does not produce acute disease in either newborn or adult *C. callosus* although animals can be infected parenterally or by the nasal route (Johnson et al., 1973). Extensive experimental studies were undertaken in the Middle America Research Unit in Panama from 1963 onwards using a breeding colony established with pairs of wild-caught *C. callosus*. Most experiments were performed with the Carvallo strain which had undergone a limited number of passages in suckling hamster brains. Inoculation of newborn animals invariably resulted in chronic infections with continuous circulation of virus in the blood and viruria. Virus was also recovered from oral secretions (Justines and Johnson, 1969). Virus replication occurred first in lymph nodes close to the site of inoculation, and thenceforth rapidly spread to infect the spleen by the third day after infection and to all other organs by day 6 (Johnson et al., 1973). Viral antigen was clearly seen by immunofluorescent staining in the cytoplasm of lymphoid cells, in the cortices of lymph nodes and the germinal centre of the spleen. Other cells clearly showed evidence of viral activity including bone marrow myeloid cells, vascular endothelium and macrophages of both the lung and liver. There was no evidence of antigen in the parenchyma of either organ, however, although large numbers of neurones and cells in the secretory glands were strongly positive. During the initial weeks of infection, the titres of virus in various organs reached 10^8 pfu/g of tissue, considerably in excess of the titre found in the blood. These levels decreased by 2 logs at 6 months postinfection together with the extent of detectable viral antigen. The growth of chronically infected newborn animals was considerably retarded; the similar observation of

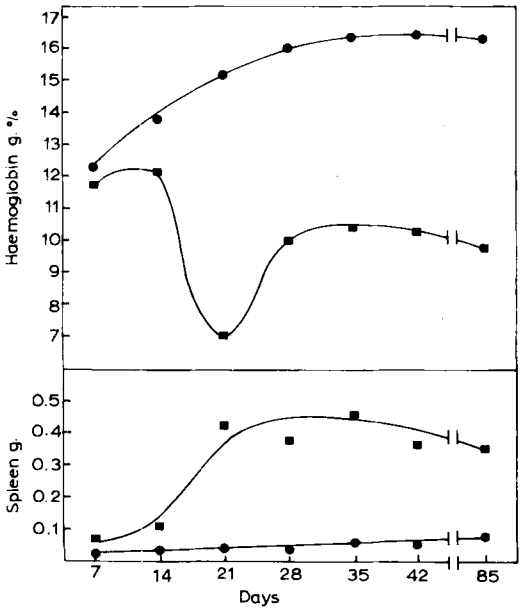

Fig. 5.2. Haemoglobin levels in *Calomys callosus* infected within 4 days after birth. •, Normal; ■, infected (from Johnson et al., 1973).

slow weight gain in mice persistently infected with LCM virus has recently been attributed to viral interference with the synthesis of growth hormone (Oldstone et al., 1984). In all other respects, the Machupo-infected *C. callosus* appeared healthy although in pathological terms had a pronounced chronic haemolytic anaemia when hepatomegaly was present (Johnson et al., 1973). Histological examination of infected spleens showed an increase in erythropoiesis and few lymphocytes. Rapid enlargement of the spleen at 3 weeks after infection coincided with a sharp drop in the haemoglobin level to half that seen in uninfected control animals. Although the erythrocyte counts later returned to near normal levels, the haemoglobin content remained low (Johnson et al., 1973; Fig. 5.2).

A split response is seen in adult animals experimentally infected with Machupo virus, with only a proportion of animals developing a chronic infection; high dilution of a hamster brain suspension resulted in about 90% of adult animals developing a persistent infection with an accompanying viraemia similar to the infection of newborns. This percentage decreased to about 50% if larger doses of virus were used (Webb et al., 1973). In the remaining group, the viraemia had terminated by 90 days

after infection and neutralizing antibody was clearly detected. This was in marked contrast to persistently infected animals, inoculated either as neonates or adults, in which neutralizing antibody could not be detected (Justines and Johnson, 1969). In some adults, circulating virus was never detected although there was abundant viral antigen seen in various organs by immunofluorescence. Shedding of virus in the urine and oral secretions was found to be prolonged after the appearance of neutralizing antibody and disappearance of virus from the blood.

This split response to Machupo virus in *C. callosus* was apparent in animals infected at or after the 9th day of age with no differences observed between males and females. However, the ratio of viraemic to responders was changed for a constant virus dose by careful cross-breeding studies, suggesting that host genetic factors may also play a role in determining the nature of the response.

Chronically infected *C. callosus* females are infertile, with extensive virus activity in the ovaries as evidenced by the presence of viral antigen in the germinal epithelium, thecal cells of follicles and even the eggs (Johnson et al., 1973). This has led to the hypothesis that Machupo virus may regulate the size of *C. callosus* populations, whereby epizootics occur among animals genetically dominant for the viraemic response when numbers increase to a level of extensive contacts between colonies. A sharp increase in the number of infertile females then results in a population crash. Johnson cites limited evidence for this view, mainly that the prevalence of virus in *C. callosus* was much higher in areas where human infections were occurring and this in turn was directly correlated with rodent density.

Junin virus has been isolated from several rodent species trapped within endemic areas (see Chapter 2) and chronic infection accompanied by continuous excretion of virus in the urine has been demonstrated by experimental infection of *Calomys musculinus* and *Calomys laucha* (Sabatini et al., 1977). A persistent viraemia invariably follows infection by the intranasal route and horizontal transmission readily occurs by placing uninfected 21-day-old animals into cages with chronically infected animals (Martinez Peralta et al., 1979a). Two of six animals in the latter study developed a chronic viraemia and in both cases a high titre of infectious virus was recovered only from the salivary glands. Although there were no histological changes, viral antigen was clearly demonstrated in the lumen and cytoplasm of secretory cells. Virus particles were clearly seen at the plasma membranes of acinar cells and in secretory spaces adjacent to the ducts.

The guinea pig has frequently been used as a sensitive biological mark-

er of Junin virus virulence, with the pathogenic XJ strain producing 100% antibody in infected animals. In contrast the attenuated XJ-Cl_3 strain produced by further passage in mice and cell cultures kills less than 20% of infected animals (Carballal, 1977; Avila et al., 1979). The XJ strain, originally isolated from a human case of Argentine haemorrhagic fever in 1958, kills adult guinea pigs 11–14 days after infection regardless of route of inoculation (Guerrero et al., 1969; Boxaca et al., 1961). The clinical disease is characterized by fever, loss of body weight and hyperthermia. Haematological alterations are marked and have been extensively studied. The total white cell count during the peak of infection decreases sharply to less than 40% of normal values, accompanied by an absolute neutropenia and lymphopenia. There is also a severe thrombocytopenia leading to a prolonged serum clotting time (Nota et al., 1969; Molinas et al., 1978). The clotting factors are reduced to approximately 10% of the normal values before infection. Fibrin monomers have been consistently found during this period, although serum levels fall in the later stages of the disease. Fibrinogen levels increase during the period immediately before death, although Molinas and colleagues have pointed out that impaired clearance of degraded fibrinogen or enhanced levels of synthesis may be responsible for this increased synthesis. Throughout these studies there was little histological evidence of fibrin deposits in affected organs suggesting that disseminated intravascular coagulation is not a feature of Junin virus pathogenesis.

Other serological features of Junin virus infection in the guinea pig include an increase in both acid and alkaline phosphatase levels (Kierzembaum et al., 1970), serum lipoproteins and beta-globulins (Budzko, 1965). The significance of these observations is unknown.

Microscopical examination of buffy coat cells from infected animals has shown extensive cytoplasmic vacuolization and lysis in up to 80% of polynuclear cells (Carballal et al., 1977a, b). Approximately 35% of circulating lymphocytes showed a similar cytopathology although no virus-like particles could be detected despite the co-existence of over 6 logs of infectious virus in the blood. Thin sections of various organs has revealed extensive necrosis of the lymphoid cells in both spleen and lymph nodes (Molinas et al., 1978). Alterations in the bone marrow are evident as early as the 5th day of infection, progressing to day 11 when over 80% of the cells in the haematopoietic series show signs of injury (Caballal et al., 1977a).

As in the case of human infection, Junin virus is immunosuppressive in guinea pigs. Some interesting differences between virulent and avirulent strains have been reported recently. Immunosuppression with either

cyclosporin A or cyclophosphamide has little effect on the virulence of wild-type virus but similar treatment of animals infected with the normally avirulent XJ-44 strain resulted in extensive virus replication and death from a progressive culminating haemorrhagic disease (Kenyon et al., 1985). The survival time was prolonged compared to animals infected with wild-type virus, however, and the later stages were characterized by involvement of the central nervous system. High titres of virus were recovered from the brains of these animals and there were associated signs of encephalitis and gliosis. The lack of immunopathology in these animals infected with either strain clearly contrasts with the classical model of LCM infection in adult mice but it remains unclear as to the mechanism whereby avirulent virus induces severe illness in immunosuppressed animals. The development of a specific antibody response may be a vital factor both in limiting the spread of virus and in the generation of cytotoxic T cells for infected cells.

The titre of infectious virus was over 100 times greater in bone marrow specimens compared to the level of infectivity in the circulation, and virus particles were clearly seen in megakaryocyte channels, suggesting that megakaryocytes may be a primary site of virus replication in the guinea pig. This is consistent with the severe thrombocytopenia induced by the virus. Neighbouring lymphoid cells showed both focal lysis of the plasma membrane and progressive condensation and necrosis although virus particles could not be seen and no viral antigen detected by immunofluorescence (Carballal et al., 1977a, 1981a). Whether these changes directly contribute to the observed immunosuppression has not been fully investigated. Guinea pigs infected with the XJ strain show depressed primary and secondary humoral responses to sheep erythrocytes (Parodi et al., 1967; Frigerio, 1977) and moribund animals show a lack of detectable antibody to the virus (Carballal et al., 1981a). However, there is no apparent change in the percentage of B cells in either organs or peripheral blood although total cell counts were much reduced (Carballal et al., 1981b). In contrast, there is a marked decrease in both absolute numbers and in percentage terms of T cells in both spleen and lymph nodes suggesting that cell-mediated immunity is specifically impaired during acute infection.

Junin virus disseminates to infect all organs of the guinea pig with the exception of the brain (Guerrero et al., 1977) although passage history and route of inoculation may affect the neurotropic potential of the virus. For instance, Boxaca et al. (1984b) found that splenic passage of the XJ strain enhanced the neurotropism of the virus when inoculated intraperitoneally. Although significant levels of virus are found in the kidneys,

infectious virus is rarely recovered in significant titres from the urine in this host. Pathological examination of this organ shows similar histological changes to those seen in human cases of Argentine haemorrhagic fever, albeit more severe. The glomerular epithelium showed swelling of the endoplasmic reticulum with dilation and convolution of the cisternae (Cossio et al., 1977). Viral antigen was clearly present both in these cells and in the tubular cells. Noticeably, immune complex deposition in glomeruli has not been found, an observation consistent with the absence of detectable viral antibody in infected animals.

Inoculation of guinea pigs with the attenuated XJ-Cl_3 strain results in the development of a mild or inapparent infection in 80% of animals, evidence of virus replication being restricted to the spleen, lymph nodes, lungs, pancreas and salivary glands (Avila et al., 1979, 1981), although attenuated virus has been demonstrated by co-cultivation methods in the bone marrow and brain (Guerrero et al., 1985). Attenuated strains show a marked neurotropic potential, probably owing to the survival of these animals beyond the acute infection stage (Boxaca et al., 1984; Kenyon et al., 1985), although repeated passage of the virulent XJ strain beyond the 30th pass also leads to a marked increase in neurovirulence (Oubina and Carballal, 1985). Despite some weight loss 3–4 weeks after infection, survivors rapidly regained weight on recovery. Even among those animals which died, symptoms of infection were minimal, with gross pathology restricted to a few cases of petechiae of the adrenals; this contrasts with the extensive haemorrhage and petechiae of organs seen at autopsy in animals infected with the virulent XJ strain. Animals surviving infection with the XJ-Cl_3 strain developed neutralizing antibody from day 13, reaching a peak titre 3 weeks after infection. The reasons for the different behaviour of these two strains are unclear; XJ_0, a strain obtained at an intermediate stage of attenuation from the parental XJ virus, gives a similar pathology in guinea pigs as the cloned XJ-Cl_3 variant (Avila et al., 1981; Boxaca et al., 1981). It has been suggested that the strain variations are related to their respective tropisms for different cells of the lymphoid system; Laguens et al. (1983a) have shown that macrophages are the main targets for virus replication in the spleens of animals infected with the virulent XJ strain, a similar observation as discussed previously for LCM virus infection of mice where most of the viral antigen can be seen to accumulate in macrophage-like cells early in infection (Lehmann-Grube and Lohler, 1981b). In contrast, Laguens and colleagues found that infection of guinea pigs with the attenuated XJ-Cl_3 strain did not result in significant changes in lymphoid tissue and attempts to detect either viral antigen or infectious virus from macro-

phages were unsuccessful. The attenuated virus could only be recovered from dendritic cells 1–4 weeks after inoculation. Virus could not be recovered from the bone marrow either, a finding contrary to previous work that this may be a primary site of virus replication in the guinea pig (Carballal et al., 1977a, 1981). Although it may be postulated that macrophages in the guinea pig do not bear the receptors for the attenuated strain, this is unlikely as both virulent and avirulent strains replicate equally well in cultured murine macrophages (Gonzalez et al., 1982). Similarly, differences in susceptibility of inbred hamster strains to Pichinde virus are not reflected in any difference in peritoneal macrophages from susceptible and resistant strains to support virus replication.

Chromosomal alterations have been seen in bone marrow smears only from animals infected with the virulent XJ strain; the frequencies of chromatid breaks, abnormal cells and fragmentation of chromosomes were significantly higher in comparison to either control animals or animals infected with the avirulent XJ-Cl_3 strain (Dulout et al., 1983). The significance of these results is as yet unclear.

Strains of Junin virus isolated from *Calomys* rodents caught in an endemic area show an intermediate virulence for guinea pigs. The MC2 strain appears also to be neurotropic with a high titre of virus being recovered from the brain of experimentally infected guinea pigs and an associated meningoencephalitis (Avila et al., 1981).

Transplacental transmission in guinea pigs has been shown both for pathogenic and avirulent strains of Junin virus (Gomez and Boxaca, 1981; Sangiorgio and Weissenbacher, 1983; Boxaca et al., 1984a) and this mode of transmission has been implicated as one of the major mechanisms whereby a reservoir of virus infection is maintained in the wild (Sabattini et al., 1977). Guinea pigs infected in the last third of gestation with the XJ strain died with a disease clinically similar to that seen in non-pregnant animals; in all cases autopsy revealed extensive haemorrhage in the foetuses, particularly in the stomach, intestines and lungs and severe congestion of the brain. Haemorrhage was also readily seen in the amniotic fluid and virus was readily recovered from the foetal tissues (Gomez and Boxaca, 1981). An interesting observation in the latter study was the increase in mortality of mothers infected prior to pregnancy; 44% of these animals died shortly after giving birth as compared to 16% in non-infected mothers. The highest mortality occurred when infection developed immediately before term. Nearly 80% of the mothers died and all of the neonates born to the infected mothers. Virus was recovered from both the spleen and lungs of neonates, albeit at low titre (Sangiorgio and

Weissenbacher, 1983). Transplacental transmission also occurs in pregnant animals infected with the avirulent XJ-Cl_3 strain of Junin (Boxaca et al., 1984a). The attenuated virus was successfully isolated from the spleen and to a lesser extent, the brains of dissected foetuses. In one instance, a mother with no signs of disease transmitted the virus to a litter in a second pregnancy, thus demonstrating that transplacental transmission may occur also in mothers chronically infected with Junin virus.

There is some evidence suggesting colostral transmission in man (Maiztegui et al., 1973). Virus has been recovered from the mammary glands of acutely infected nursing guinea pigs and transmission to uninfected litters was found by Sangiorgio and Weissenbacher (1983); 10 of 12 animals died during the suckling period between 7 and 38 days after birth. Although the number of survivors was very small, there was no evidence of chronic infection and both animals were susceptible to challenge with pathogenic virus some weeks later. Taken together, these results indicate that transplacental transmission may occur in the other hosts and that the similar placental composition of the guinea pig to that in man makes the guinea pig a suitable model for further studies.

As with LCM virus, Junin is known to infect and replicate in many tissues of infected hamsters, in particular the liver, lung and thymus. A detailed study of infant hamsters was reported by Bruno-Lobo et al. (1968). Initially, virus was also found in lymph node lymphocytes and may have provided a continuing source of virus into the circulation, although viraemia levels were consistently less than those observed in visceral organs. This suggests that macrophage clearance continued to operate during acute infection despite the probable infection of a proportion of macrophages within infected tissues.

After day 4, considerable evidence of virus infection was found in small capillaries and cells of perivascular connective tissue, in particular the meninges of the brain, and suggests that capillary epithelium is a major site of Junin virus replication (Andrews et al., 1978). Similar localization of virus activity within small vessels has also been reported for arboviruses, e.g. West Nile (Kundin et al., 1963) and Sindbis (Johnson, 1965).

The spread of Junin virus to the liver parenchyma appears to be limited, with viral antigen present in small foci presumably as a result of virus spread from infected Kupffer cells. The limited extent of hepatitis in Junin-infected hamsters is reflected by absence of elevated transaminase levels and no histological abnormalities. In contrast, Junin virus enters the central nervous system resulting in extensive neuropathology and infection of neurones in many areas of the brain. Capillary fluorescence

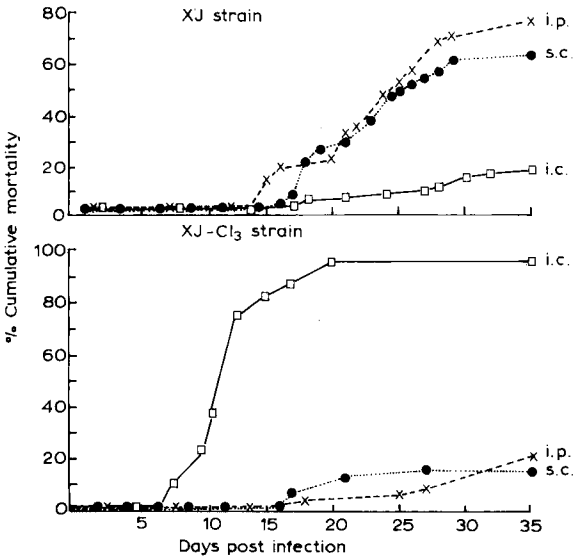

Fig. 5.3. Mortality rates of 2-day-old rats inoculated by various routes with either the XJ or XJ-Cl_3 strains of Junin virus. i.c. = Intracerebral; s.c. = subcutaneous; i.p. = intraperitoneal (from Avila et al., 1981b).

was clearly present 6 days after infection in the leptomeninges. Progressive involvement of the nervous tissue was seen, with viral antigen-positive foci of infected cells spreading to involve the medulla, hippocampus and the cerebral and cerebellar cortices, factors that may have clearly contributed to the mortality of these animals. Noteworthy was the absence of any significant histological changes in either the central nervous system or other organs supporting extensive virus antigen production. In one instance, viral antigen was detected in the brain of a surviving animal as long as 45 days after infection, a finding which may be of relevance in understanding chronic infection in the hamster model.

Wistar rats are also susceptible to Junin virus (Nejamkis et al., 1977) although the outcome of infection is dependent on both the laboratory strain of virus used and the age of inoculated animals (Avila et al., 1981b). Animals infected with a strain pathogenic for man (XJ) before the age of 2 days are largely resistant to the infection. Susceptibility increases from 8 to 12 days when mortality is over 90%, and then declines rapidly until adult animals are resistant. However, susceptibility to the non-pathogenic XJ-Cl_3 strain is greater immediately after birth until day 12 (Fig. 5.3). This is the reverse of the outcome of XJ-Cl_3 infection of guinea pigs, where the animals are largely resistant to infection by

the attenuated strain. Two day old rats infected intracerebrally with either strain showed similar levels of replication in the brain despite the difference in mortality between the two groups. The reason for this is unclear, however, and clarification of the rat as a model for studying Junin virus pathogenesis is awaited. The high mortality in very young animals inoculated with the XJ-Cl_3 virus however, appeared to be dependent on the route of inoculation, as mortality dropped from 100% to below 20% if given either subcutaneously or intraperitoneally.

5.1.4. Other arenaviruses

The Tacaribe complex contains a further six viruses non-pathogenic for man, all of which serologically cross-react to varying degrees with Tacaribe virus, the type member of the group (Wulff et al., 1978). This virus is unique among the arenaviruses in that its original isolation in Trinidad was from *Artibeus* species of fruit-eating bats with no record of infection among the local rodent population (Downs et al., 1963). Casals and colleagues have examined in some detail the virulence of Tacaribe complex arenaviruses in newborn mice (Mettler and Casals, 1970, 1973). Newborn mice inoculated intraperitoneally with increasing dilutions of these viruses showed that Tamiami virus was of relatively low virulence whereas Junin, Amapari, Pichinde and Tacaribe caused severe mortality even at high dilutions. Hind-limb paralysis was noted between 9 and 28 days postinfection in animals surviving infection with Pichinde, Junin or Tacaribe viruses (Table 5.1). With the exception of Pichinde-infected animals, all groups possessed CF antibodies by day 30.

In contrast to LCM virus, laboratory studies have shown that Tacaribe virus is uniformly lethal for neonatal mice but adults survive (Borden and

TABLE 5.1.
Response in newborn mice to intraperitoneal infection with Tacaribe complex arenaviruses (data from Mettler and Casals, 1970, 1973)

Virus	% Survivors on day 30	Hind-limb paralysis?	CF antibodies?
Tamiami	89.4	No	Yes
Amapari	33.8	No	Yes
Pichinde	33.1	Yes	No
Tacaribe	24.4	Yes	Yes
Junin	•	Yes	Yes

• Data not given.

Nathanson, 1974). Moribund newborn mice show pronounced paralysis and ataxia, but rarely show the convulsive diatheses typical of lethal LCM infection (Downs et al., 1963; Borden et al., 1971). The lesions observed in Tacaribe-infected animals are similar to those observed with Junin virus, consisting of choroiditis, glial hyperplasia, vasculitis and perivasculitis accompanied by extensive lymphocytic infiltration. This pathology is absent in thymetomized, immunosuppressed or genetically athymic (nude) mice (Parodi et al., 1970; Besuschio et al., 1973; Weissenbacher et al., 1982), indicating that thymus-dependent cellular immune responses play a major role in pathogenesis.

Passive transfer of anti-lymphocytic serum to infected newborn mice prolongs the median survival time (Borden et al., 1971; Borden and Nathanson, 1974), suggesting that Tacaribe virus-induced disease in newborns may be immune-mediated. The prolongation of survival was accompanied by body weight loss and continued presence of viral antigen in both brain and visceral tissues. All of the survivors treated with anti-lymphocytic serum became carriers of the virus, with infectivity titres in excess of 10^5 LD_{50} per gramme of brain tissue.

Although immune-mediated disease may be a common feature in both LCM and Tacaribe infection of mice, the distribution of virus and viral antigens differ appreciably, probably reflecting different tissue and cellular tropisms of these two viruses which in turn leads to a distinct clinical picture and pathology. Histologically, lethal Tacaribe virus infection is accompanied by necrotic lesions of the cerebellum, cerebral cortex and hippocampus (Borden and Nathanson, 1974). In contrast to LCM infection of adult mice, only a minimal involvement of the leptomeninges was seen and choroiditis was absent in this study. These findings correlated well with the visualization of Tacaribe specific viral antigens as detected by immunofluorescence, although a heavy burden of viral antigens was also seen in the subependymal plate which appeared histologically to be normal. Adoptive transfer experiments with Tacaribe immune spleen cells shortened the survival time, an effect also seen with LCM infection of adults (Gilden et al., 1972b). This is accompanied by an enhanced severity of parenchymal cell necrosis. In contrast, passive immunization of infected animals with antibody to Tacaribe did not significantly change the pattern of virus-induced mortality.

The pathogenicity of both Junin and Tacaribe viruses decrease with increasing mouse age (Boxaca et al., 1973; Borden et al., 1971; Coto and Leon, 1978). Borden and Nathanson (1974) found that animals infected at 10–14 days of age succumbed only to a brief illness characterized by ruffled fur, hunched postures and occasional tonic-clonic seizures. These

authors could find no evidence of infection when animals were inoculated 60 days or later after birth, and there was no sign of virus persistence in either inoculated adults or the few survivors among infected newborn or suckling animals. Coto and Leon (1978) have also examined in some detail the relationship between age of mice inoculated intracerebrally with Tacaribe virus and mortality. A decreased level of mortality was observed with increasing age; this was paralleled by a shortening in the mean survival time of those animals which eventually died. Superimposed upon these results was the finding that the 30% level of mortality among mice inoculated 30 days of age with 125 LD_{50} of virus could be increased to 60% by using a 2-log increase in virus dose. Among the survivors at various ages receiving higher doses of Tacaribe virus there appeared to be signs of virus persistence with stunting of growth and loss of fur quality. Coto and Leon further observed that the reduction in the mean survival rate with age was paralleled by a general decrease in the period of clinical illness and that unlike mice over the age of 12 days was unrelated to virus age. These differences appeared to be unrelated to virus growth kinetics and were seen in the brains of mice infected either before 2 days of age or 10 days later. Borden and Nathanson (1974) have proposed that the paradox of increased pathogenicity with age may be explained by a gradual decrease in the number of cells permissive for Tacaribe virus coupled with an increase in the responsiveness of the host immune defence mechanisms. However, the results of Coto and Leon would suggest that this enhanced reactivity to the virus immediately prior to the rapid decline in mortality may be related to the appearance of host factors adjacent to, or preceding the development of the immune response.

Although signs of persistent Tacaribe virus infection have been reported (Coto and Leon, 1978), the apparent general failure of the virus to induce chronic infections may be at least in part due to the lack of virus tropism for cells of the lymphoid system, thereby preventing the occurrence of a transient immunosuppression as the host's immune system matures (Borden and Nathanson, 1974). Also, it has been pointed out by the latter authors that Tacaribe viral antigens are apparently more immunogenic as compared to LCM virus, as evidenced by the development of inflammation even in newborn animals. However, further work would be required to substantiate this hypothesis; the precise nature of the viral antigenic determinants involved could be usefully dissected with the recently available monoclonal antibodies to these proteins, as has recently been achieved in the study of LCM virus persistence *in vivo* (Oldstone et al., 1982). Tacaribe virus-infected mice treated with anti-

lymphocytic serum become virus carriers for life (Borden et al., 1971). Histopathological signs of infection were absent, yet over 7 logs of virus were recovered from the brains 19 days after infection. Although in many respects this finding resembles the effect of anti-lymphocytic serum on the pathogenesis of LCM infection in mice, the host-virus relationship may be fundamentally different in animals infected with Tacaribe virus. For example, the inflammatory response in untreated animals was shown by Borden et al. to be focal and not involving the ependyma, meninges or parenchyma of the brain. This may partly be explained by variation in tissue tropism, mediated by the unique properties of the virus and/or hitherto undefined host genetic factors. That the virulence of Tacaribe virus for adult mice may be enhanced has been shown by Rosato et al. (1978) who successively passaged the virus in adult mice. Intracerebral inoculation of such variants into mice 75–100 days of age resulted in a progressive encephalitis. Animals developed ruffled fur, ventriflexed posture, paralysis and eventual death 10–14 days after infection. Histological examination revealed a moderate to severe meningoencephalitis largely restricted to the hippocampus and accompanied by segmented necrosis of neurones in the hippocampal gyrus. The immunopathological nature of the disease in adults was confirmed by the apparent absence of a response in athymic mice. The increase in virulence reported by Rosato et al. was not sustained at high passage levels, however; although the reason for this was unclear, a similar loss of infectivity on repeated passage was also reported by Downs et al. (1963).

Almost all thymectomized mice survive infection with Junin, Machupo, Tacaribe or Pichinde viruses (Parodi et al., 1970c; Besuschio et al., 1973) Amapari virus is apparently an exception, in that athymic mice were as equally susceptible to infection as their normal littermates. Thymectomized animals infected with Amapari virus died with neurological symptoms between 10 and 18 days after infection with various brain lesions, including choroiditis, glial hyperplasia and perivascular cuffing. Although these observations suggest that Amapari-related disease may be unique and independent of T cells, this has not subsequently been investigated further using genetically athymic (nude) mice in the same manner as in the case of Junin virus (Weissenbacher et al., 1982). Besuschio et al. speculated that tissue damage may occur in the brain of Amapari virus infected animals thymectomized at birth owing to the activity of a subpopulation of T cells which mature before birth but evidence is not available to clarify this possibility.

Pinchinde and Tacaribe viruses can be differentiated from Amapari and Tamiami viruses by peripheral inoculation into 1- or 2-day-old mice (Mettler and Casals, 1973). Both Pichinde and Tacaribe viruses induce a

paralytic sequelae in surviving animals 9–28 days after infection that may affect either one or both limbs. This was not observed in mice surviving Amapari or Tamiami virus infection by the intraperitoneal route. Tamiami virus was noticeably less virulent for mice in contrast to the other three viruses included in this study.

A detailed study of Tamiami virus infection of its natural host the cotton rat (*Sigmodon hispidus*) has been reported by Murphy et al. (1976). The major features of this virus-host system are long lasting humoral immunity coupled with a particularly slow route of virus elimination. Indeed high titres of infectious virus were recovered from organs at times after infection when circulating antibody was clearly demonstrable. In animals infected 2 days after birth, viral antigen, as detected by immunofluorescence first appeared in the lymph nodes, thymus, spleen and bone marrow, reaching peak titres within 2 weeks after infection. Pathological features in these tissues included extensive infection of reticuloendothelial cells and a proportion of macrophages, although light microscopy gave no indication of viral cytopathology. Although few virus particles or inclusion bodies were seen by electronmicroscopy except in bone marrow megakaryocytes, intense granular immunofluorescence patterns in lymphoreticular tissues indicated extensive virus activity. Subsequent infection of the liver never exceeded 30% of hepatocytes and liver architecture was preserved. The involvement of the submaxillary gland was also apparent at this time. Between 3 and 6 weeks after infection viral antigen was present in kidney, adrenals, bladder and respiratory tract. By 2 months antigen was detected in approximately half of the kidney tubules although only weak immunofluorescence was seen in the glomerular regions. Although high titres of both complement-fixing and neutralizing antibody were present by the end of the fourth week, there was no evidence of significant immune-complex deposition in the kidney. Of particular interest was the finding of large amounts of antigen in bladder epithelium; at no time did the titre of virus exceed that in the blood and virus was never recovered in the urine.

Tamiami viral antigens were detected in brain tissue from 2 weeks, and progressively developed until approximately 10% of neurones gave positive reactions. The presence of viral antigen coincided with inclusion bodies visible by electron microscopy, the latter occurring most frequently near the edge of neuronal cytoplasm. Again, there was no obvious cytopathology or evidence of inflammation, and antigen persisted in brain tissue for up to one year.

Hamsters have been examined as an alternative host of Pichinde virus (Buchmeier and Rawls, 1977; Murphy et al., 1977). Differences were

found in the susceptibility of two different strains of Golden Syrian hamsters (*Mesocricetus auratus*). The virus normally produces a lethal disease in newborn animals of both LVG and MHA strains; indeed all members of the Tacaribe complex normally produce lethal infections in newborn hamsters. However, infection of adult animals does not normally induce a lethal infection, and LVG strain hamsters became resistant by 7 days of age. In contrast, MHA hamsters remained susceptible as adults with mortality approaching 100% after intraperitoneal inoculation of 4 logs of virus or more. Adult MHA hamsters were unable to limit virus replication as effectively as the resistant LVG strain, with over 5 logs more virus being present in the serum. The major organs infected were the liver, spleen and kidneys. Viral antigen appeared in the liver of MHA strain hamsters 4 days after infection after which extensive hepatocellular necrosis developed in the absence of a significant inflammatory response. Electron microscopy showed extensive viral activity in both infected hepatocytes and adjacent Kupffer cells, a finding consistent with a high content of viral antigen. Alterations of the spleen included extensive necrosis of both white and red pulp with destruction of the cordsinus in large areas. Viral antigen was seen by immunofluorescence in both the red pulp and in marginal zones by day 10 with electron microscopy indicating macrophages to be the primary cell target in the red pulp. Intracytoplasmic vacuolation, degeneration of mitochondria and plasma membrane dissolution were visible and arenavirus particles were present in adjacent extracellular spaces. Murphy and colleagues draw attention to the early lymphotropism of many arenaviruses and in this respect Pichinde virus is no exception; however, infection of the MHA hamster strain is characterized by a particularly severe reticuloendothelial necrosis greater than that previously found. This was particularly evident in the spleen in both strains of hamster examined, but only in the MHA strain animals did this necrosis appear to continue unabated, spreading progressively into the red pulp from the marginal zones. Initial involvement of macrophages may explain the pathology of the infection; noticeably peritoneal macrophages from both LVG and MHA strains supported replication of Pichinde virus equally well. However, the marked tropism of the virus for the liver and spleen *in vivo* may reflect minor differences between cells of the macrophage series localized in different tissues. Interestingly, the lymph nodes and other lymphoid organs were spared the extensive necrosis seen in the spleen, possibly reflecting the fewer numbers of macrophages in an active state in these organs. The general absence of inflammatory responses, the failure of immunosuppression to moderate lethal infection in MHA strain animals and the

increase in mortality of LVG strain hamsters receiving cyclophosphamide together indicate the direct cytopathic effects of the virus are responsible for disease in this host. Clearly, host genetic factors may play a role in determining the outcome of infection, with MHA strain being deficient in co-operation between different compartments of the cellular immune system. This could possibly be due to a dysfunction of macrophages in limiting virus replication or the presentation of viral antigens at the cell surface. An alternative explanation is that the MHA strain of hamster possess a larger number of splenic target cells for the virus with properties similar to natural killer cells (Gee et al., 1981). The high mortality seen in MHA strain hamsters appears to be dependent on the route of inoculation; these animals survive if infected via the footpad, with virus spreading to the lymph nodes and eventually the spleen with peak titres of replication 5 days after infection. This is much slower compared to infection by the intraperitoneal route and allowed the animal to restrict the extent of virus replication. However, intermediate passage of the virus to the spleen via the popliteal lymph nodes showed a greater proportion of natural killer-like cells infected in comparison to the resistant LVG strain. The reason for this is unclear although at least two phenotypic differences between hamster strains may be responsible for a specific deficiency in a T cell subset (Gee et al., 1981) although the nature of this has yet to be defined.

5.2. Experimental infections of primates

5.2.1. Lymphocytic choriomeningitis virus

The pathology of LCM infection in the brains of infected monkeys has been found to be variable, even between individual animals infected in any one series of experiments (Lillie, 1936). Infiltration of the choroid plexus varied from foci containing lymphocytes only to diffuse inflammation consisting of lymphocytes, plasma cells and macrophages. Meningeal inflammation was less severe and widely distributed over both brain and spinal cord. A high grade fever developed 3–10 days after infection, but generally in these studies the animals recovered (Armstrong and Lillie, 1934; Rivers and Scott, 1936a). This is in contrast to other studies, particularly in rhesus monkeys, where a more severe inflammatory reaction similar to that seen in mice was found and resulted in high mortality of infected animals (Findlay and Stern, 1936; Milzer and Levinson, 1949; Danes, 1963, quoted in Lehmann-Grube, 1961).

The study of Danes and colleagues is particularly interesting as the dose levels required to produce disease by inhalation were determined for the closely related cynomolgus and rhesus monkeys. The latter were found to be exquisitely sensitive, a lethal dose requiring only 12 mouse LD_{50} units of LCM virus; in cynomolgus monkeys, a dose of less than 200 LD_{50} produced clinical disease and viraemia and more than 350 LD_{50} units were found to be lethal.

Lehmann-Grube (1971) draws attention to the description of disease in monkeys originally thought to be due to distemper virus but subsequently identified as being LCM virus infections (Dalldorf, 1938). A distinct biphasic febrile illness was observed in half the animals followed regardless of route of inoculation. In addition, severe weakness and emaciation were seen together with diarrhoea. The range of symptoms and pathology of LCM infection in primates thus indicates that some variation in response in susceptibility of different monkeys to Lassa virus may thus be anticipated.

5.2.2. Lassa virus

First attempts to study Lassa virus infections in non-human primates were performed using squirrel monkeys *(Saimiri scirreus)*. Intramuscular inoculation of animals resulted in essentially a pantropism for all the major organs examined after an incubation period of 8–18 days (Walker et al., 1975). There appeared a close connection between the severity of disease and the extent of virus replication in the various organs, with the liver, kidneys and lymph nodes being particularly affected, although this study consisted of only four animals. High titres of virus were found in the urine of one animal examined, a feature together with a viraemia clearly resembling acute infection of man. Histological examination showed evidence of cellular degeneration despite the general absence of gross pathological lesions. Walker and colleagues particularly noticed that cortical follicles of lymph nodes were necrotic and contained multinucleate cells. Necrosis of the periarteriolar lymphocytic sheath of the spleen was also affected, and the authors speculated that this may have resulted in B cell depletion and a delayed humoral antibody response to viral antigen. The observed hyperplasia of the paracortical regions of infected lymph nodes may indicate an active T cell response to the infection, but the abnormally extensive sinus histiocytes may obstruct flow in the lymphatic system. The authors suggest that this may account for the oedema and effusion seen in human cases, an hypothesis that could equally be extended to Junin and Machupo infections.

The pathology of Lassa virus infection in the rhesus monkey *(Macaca mulatta)* has been extensively documented (Jahrling et al., 1980; Callis et al., 1982; Walker et al., 1982). The highest titre of infectious virus was recovered from the liver, although substantial amounts of virus were recovered from the lung, adrenal, pancreas, spleen, kidneys and lymph nodes. These values often exceeded 7 logs per gramme of tissue and the high levels of virus replication in infected animals may account for the finding of virus additionally in the brain and spinal cord. Of 17 animals infected with 10^5 pfu of the Josiah strain of Lassa virus by Jahrling et al., nine succumbed to the infection with clinical signs similar to those seen in human cases. These animals became lethargic and febrile 4 days after infection followed some days later by a conjunctivitis and a skin rash. Death occurred in this group of animals on average 11 days after infection. In general, symptoms developed some days later in those animals surviving the infection with fever being intermittent and variable in duration.

Acute Lassa infection of primates develops sequentially as phases of cellular necrosis, host responses to infection and lastly tissue regeneration. Hepatocellular necrosis was a consistent finding in all animals and the development of a viraemia coincided with an elevation in serum transaminase levels, a marker of hepatitis injury (Callis et al., 1982), but did not involve more than 10% of hepatocytes (Walker et al., 1982). The necrotic foci were randomly distributed and multifocal accompanied by a slight infiltration of mononuclear cells. The cytoplasm of individual hepatocytes was acidophilic and found to have large amounts of viral antigen and were randomly distributed throughout the parenchyma (Jahrling et al., 1980; Walker et al., 1982). The spleen in all animals showed an increase in the numbers of mononuclear cells although viral antigens were absent from the white pulp. The lymph nodes showed areas of lymphocyte depletion although again large infected cells of the mononuclear series were detected by immunofluorescence. Other organs with histological signs included the kidneys, adrenal glands and lungs. An interstitial pneumonia was frequently seen characterized by alveolar wall thickening and inflammation. A mild inflammation was also seen in the kidneys and brain but each was restricted to the blood vessels. Inflammation in the choroid plexi was evident but not nearly so prominent a feature as seen in non-human primates infected with either Junin or Machupo viruses although notably more prominent than that seen in human Lassa fever. Generalized vascular damage was a characteristic feature in all infected animals and probably is more extensive than in human infections (Walker et al., 1982).

The rhesus monkey provides a more useful laboratory model of Lassa fever compared to either experimentally infected rodents, guinea pigs or squirrel monkeys. The reproduction of the hepatotropism of this virus in the rhesus monkey model is particularly interesting, a feature of this virus which clearly distinguishes it from Junin and Machupo viruses. The parallel appearance of virus and transaminases in the circulation together with the minimal inflammatory response indicates that this virus may be directly cytopathic, although this remains to be proven. Callis et al. (1982) point out that an analogy may be made between Lassa virus infection of hepatocytes and LCM infection of neuroblastoma cells where infection leads to loss of so-called 'luxury functions' without necessarily affecting the normal physiological processes essential to the cell's survival (Oldstone et al., 1977). If such is the case in Lassa fever, viral infection of the liver may be a cause of death by preventing the synthesis of vital metabolites despite the mild lesions seen on histological examination. Such an effect may not necessarily be restricted to the liver, with high levels of virus replication clearly being seen in other organs such as the pancreas and adrenal glands.

5.2.3. Machupo and Junin viruses

Experimental Machupo virus infection of non-human primates has received considerable attention following the initial identification of the virus. For example, Webb et al. (1967) reported that marmosets *(Saguinus geoffroyi)* were susceptible following subcutaneous inoculation of the Carvallo spleen strain of the virus. Animals were equally susceptible to virus passaged through hamsters, mice or cell culture with an approximate relationship between the dose and median day of survival. Although no detailed pathology was reported in this study, all acutely infected marmosets showed anorexia and general lethargy although remaining afebrile. Virus was recovered from various organs at the time of death, including the brain, liver and kidneys. Infectious virus was grown from throat swabs taken immediately prior to death, and readily recovered from the spleen of infected animals. Successful transmission of the virus also occurred by application of a virus suspension to scarified skin or the cornea. Animals were not infected by administering the virus by either the oral or intranasal routes.

Bolivian haemorrhagic fever in rhesus monkeys *(Macaca mulatta)* is characterized by severe epithelial necrosis of the skin, gastrointestinal tract, liver and adrenal glands (Terrell et al., 1973). In the latter study, 12 animals received either 10^5, 10^3 or 10^1 pfu of the Carvallo strain of

Machupo: all the animals receiving 10^3 or more infectious units died within 20 days, there being a correlation between dose and median day of death. Haemorrhagic manifestations were found but not considered by Terrell et al. as the major cause of mortality with only one animal showing extensive petechiae at the time of death. Many animals were found to be jaundiced and all animals with late neurological symptoms possessed significant levels of serum neutralizing antibody; it remains to be determined if this plays any role in the cellular inflammatory response, e.g. by mediating antibody-dependent cellular cytotoxicity against cells which continue to harbour virus or viral antigens late in infection. A parallel may be drawn here with the observed late onset of neurological symptoms in cases of human Argentine haemorrhagic fever treated with convalescent plasma (Maiztegui et al., 1979; see Chapter 10).

Alternative primate models of Machupo infection have been described. For example, the African green monkey *(Ceropithecus aethiops)* is equally susceptible to the virus (Wagner et al., 1977; McLeod et al., 1978). Again, hepatic necrosis was a consistent finding although fewer animals exhibited neurological signs with a late encephalitis only being found in one of six animals infected. Lesions were most severe in the brain stem of this animal, characterized by lymphoreticular vasculitis and cuffing with gliosis. Extensive necrosis of the small intestine, adrenal cortex and lymphoid tissues was a common feature resembling the previously described pathology of the infection in rhesus monkeys. It should be noted that in neither case was evidence obtained for disseminated intravascular coagulation although fibrin thrombi were occasionally seen.

The New World marmoset *(Callithrix jacchus)* has recently received attention as a possible model of Argentinian haemorrhagic fever (Weissenbacher et al., 1980a; Gonzalez et al., 1983). Animals were infected intramuscularly with the XJ strain of Junin virus and sacrificed at various times up to 24 days later. All animals developed a severe disease, beginning on day 15 with anorexia, loss of body weight and signs of CNS involvement. Unlike man, however, infected marmosets did not show body temperature elevation during the acute phase of the disease. During the third week of infection, infected animals developed a progressive leucopenia, thrombocytopenia and anaemia accompanied by haemorrhage, the latter being prominent in the peritoneum, gums and pharynx. Detailed haematological findings in this animal model have been reported by Frigerio et al. (1982). Anaemia developed 7 days after infection with haematocrit and red cell counts dropping to approximately 40% of normal values over a 2-week period of illness. The authors recognized two distinct phases in the haematological changes. The first, from 7 to 14

days after infection, was characterized by an increase in the numbers of circulating reticulocytes and erythroblasts, possibly arising out of haemorrhage or damage to the bone marrow as a result of the infection. In the second period, the erythrocyte count dropped rather more sharply with a partial or total absence of reticulocytes and erythroblasts, indicating hypoplastic or aplastic anaemia. Limited examination of bone marrow smears showed features consistent with this hypothesis. In many respects, these changes resemble those seen during acute infection of guinea pigs (Guerrero et al., 1977) and man (Rugiero et al., 1959), although in man the depression in haematocrit readings are not nearly so severe. Although no other gross morphological changes were seen, microscopical lesions were present in the brain, lungs, liver, lymphatic organs and bone marrow. The lesions of the lymphoid tissue characterized by lymphocytic depletion of the cortical areas together with changes in the bone marrow closely resembled the pathology of human infection. In contrast, the interstitial pneumonia and the meningoencephalitis and vasculitis seen in the brains of infected marmosets does not parallel the pathology of infection in man. Tetanus-like convulsions may occur coinciding with high titres of virus in the brain (Weissenbacher et al., 1980a). An extensive lymphocytic infiltration seen in the brain of infected animals together with the late onset of neurological symptoms suggest that the lesions are due to immunological attack, although the extensive distribution of cells positive for viral antigen does not exclude a pathology due to the virus alone. This study noted extensive macrophage involvement with many cells positive by immunofluorescence; extensive infection of other cells in lymphoid tissue would indicate infection may be accompanied by suppression of at least some component of the host immune defence mechanisms.

Not all species of New World monkeys appear equally susceptible to Junin virus infection. Weissenbacher et al. (1978a) reported that the virus was essentially avirulent in howler monkeys *(Alouatta caraya)*. Infection was asymptomatic and a viraemia was not detected. However, inoculated animals seroconverted readily to the virus with the production of neutralizing antibody, indicating a mild infection in this species. No evidence was obtained of pathological lesions specific to the virus infection. The XJ strain of Junin virus has been shown to be pathogenic for *Cebus* monkeys, however, with a viraemia, leuco- and thrombocytopenia developing one week after infection (Carballal et al., 1980a). All the animals recovered from acute infection and developed viral antibody although one animal showed prolonged signs of central nervous system involvement manifested by limb tremors, excitability and photophobia.

Although virus was not recovered from the organs of this animal when sacrificed 77 days after infection, there were clear signs of immune complex deposition in the capillary walls of the nervous system. A similar observation was also made in a *Cebus* monkey infected with XJ-Cl_3 attenuated strain of Junin virus; in this case other symptoms included a moderate leucopenia in the absence of detectable viraemia.

CHAPTER 6

Ultrastructure of arenaviruses and infected cells

The morphology and morphogenesis of all arenaviruses are remarkably similar, with the result that it is not possible to distinguish any individual family member either by application of morphological criteria or by the appearance of virus-related structures seen within infected cells (Murphy and Whitfield, 1975). The first comparative study, performed by Murphy et al. (1969) found striking morphological similarities between Machupo, Tacaribe and LCM viruses, confirming previously reported biological and physical similarities between Machupo and LCM (Justines and Johnson, 1969; Webb, 1965). All contained 1–10 electron-dense granules approximately 20 nm in diameter with a well-defined unit membrane, giving a distinct 'sandy' appearance in thin sections. This granularity prompted the use of the name arenavirus by derivation from *Arenosus* (L = sandy), a name 'chosen to reflect the characteristic granules seen in the virion in ultrathin sections' (Rowe et al., 1970b). The name was modified soon after to arenavirus in order to avoid possible confusion with members of the adenoviridae. The presence of intravirion particles together with an apparent excessive pleomorphism clearly distinguished arenaviruses in early studies from retroviruses, many arboviruses and other membrane-bound viruses (Rowe et al., 1970b; Murphy et al., 1969; Abelson et al., 1969).

6.1. Appearance in thin sections

Cell cultures infected with arenaviruses manifest distinctive intracytoplasmic inclusion bodies. These inclusions have been described as consisting of a moderately electron-dense, smooth matrix within which are embedded numerous dense granules similar to the ribosome-like structures seen within maturing and extracellular virus particles and are clearly distinguishable from glycogen granules (Murphy et al., 1970). During the course of infection, the inclusions become progressively denser and assume smoother margins paralleling closely the development of inclusion bodies detected by light microscopy (Buckley, 1965; Abelson et al., 1969; Buckley and Casals, 1970). Virus-specific antigen was found by Abelson et al. (1969) to be closely associated with those structures seen in LCM-infected 3T3 cells, a finding that has recently been confirmed by Rodriguez et al. (1983) who found LCM-specific nucleocapsid antigen in association with host ribosomes within neurones of persistently infected mice. The changes in the fine structure of infected cell cultures precede gross cytopathological alterations, when organelle destruction and membrane breakdown occur together with a general condensation of the cytoplasm. Although arenavirus replication is known to require an intact cell nucleus, there is no apparent morphological change in nuclear structure, and nuclear inclusion bodies are absent.

Arenavirus envelopes are formed from the plasma membranes of infected cells and rarely from intracytoplasmic membranes into vacuoles. During virus maturation there is a clear increase in staining density of both leaflets of the membrane bilayer at the site of viral budding (Fig. 6.1). Insertion of viral glycoproteins appear to precede morphological changes as demonstrated by the binding of specific antibody to unaltered plasma membrane early in infection (Mannweiler and Lehmann-Grube, 1973). As the particle forms by extrusion, surface projections become visible and ribosome granules may become regularly arranged immediately below the thickened membrane. Linear filaments less than 20 nm in diameter linking these granules have been described (Murphy and Whitfield, 1975), although a direct demonstration that these represent nucleocapsid structures is still lacking.

There is a significant difference in morphology and ultrastructure between virus examined in thin sections of infected cell cultures and tissue taken from infected humans or animals at necropsy (Maiztegui et al., 1975; Speir et al., 1970). The demonstration by electron microscopy of arenavirus particles in human tissue, particularly in the liver, has been of value in confirming the viral specificity of those lesions observed by light microscopy. In Lassa fever, most particles are seen where hepatocyte

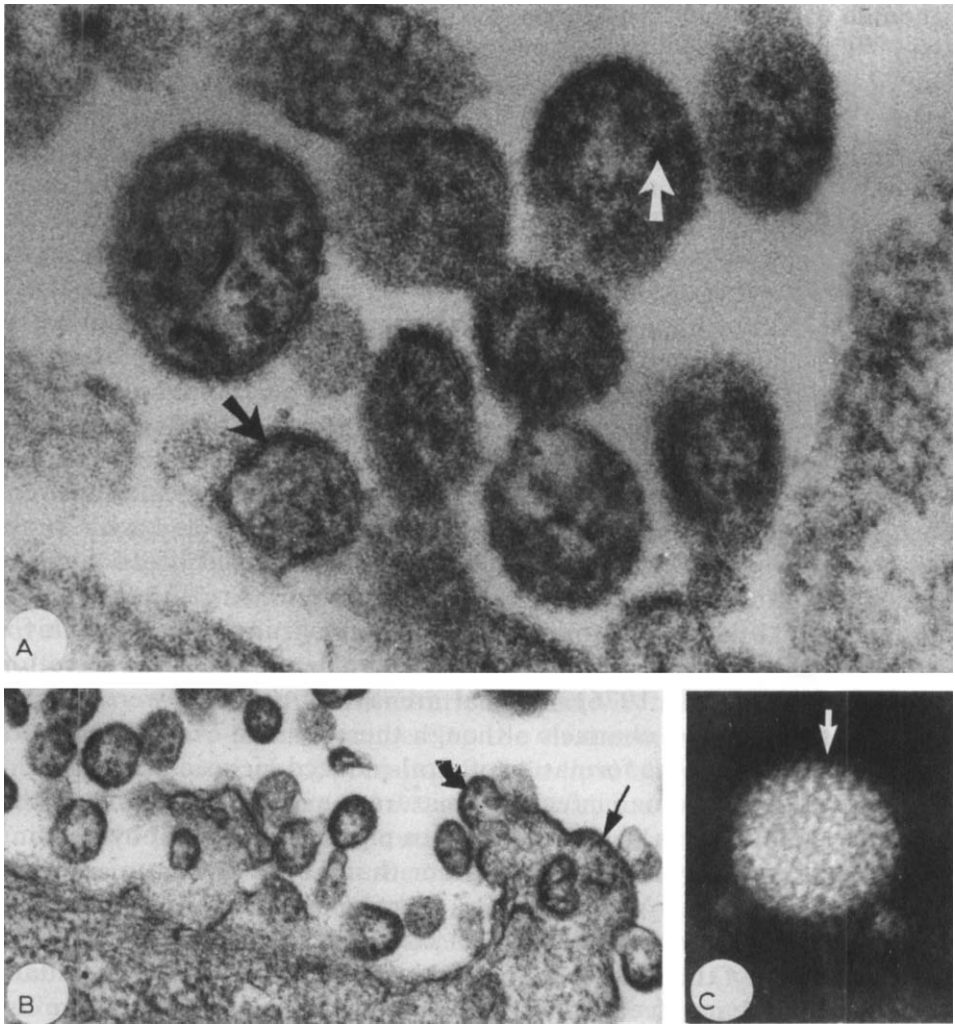

Fig. 6.1. Ultrastructure of Lassa virus and infected cells. Virus released from infected Vero cells are shown in thin section (panel A). Incorporated host cell ribosomes (white arrow) are shown within a particle surrounded by a lipid bilayer membrane (black arrow). Osmiophilic thickening of the host cell membrane occurs below which are assembled cytoplasmic ribosomes for inclusion into progeny virions (panel B). The curved arrow shows the next stage in the budding process as the membrane rounds up to enclose the internal nucleocapsid and ribosomes. Negatively stained Lassa virions show a surface covered with projections (panel C). Magnification: x 173 000 (panel A) x 47 000 (panel B) and x 282 000 (panel C). Micrographs by courtesy of Dr. D.S. Ellis.

damage is less severe and rarely in areas of extensive hepatocellular necrosis (Winn and Walker, 1975). Virus particles are also seen within

the bile canaliculi and maturation from infected hepatocytes is accompanied by thickening of membranes adjacent to the perisinusoidal or intracellular spaces between hepatocytes. Individual cells show dilation of the endoplasmic reticulum together with the accumulation of electron-dense material within the mitochondria although the extensive matrices containing ribosomal aggregates appear to be absent.

Carballal et al. (1977) found in guinea pigs experimentally infected with Junin virus that extracellular accumulations of virus particles were low and virus was rapidly transported away from infected bone marrow tissue via extracellular channels. Again, there was no evidence of inclusion bodies consisting of ribosomal aggregates. Virus activity was associated with lesions of the reticular monocytes and surrounding cells exhibiting focal lysis of the plasma membranes, cytoplasmic condensation and necrosis (Carballal et al., 1981a). These ultrastructural changes were also found in lymphatic tissue and are in accord with similar findings in tissue obtained at necropsy from patients infected with Junin virus, and give further support to the role of direct virus infection in the reticuloendothelial system in the pathogenesis of Argentine haemorrhagic fever. Likewise, Tamiami virus, an arenavirus non-pathogenic for man has a tropism for megakaryocytes and mononuclear cells in cotton rats (Murphy et al., 1976). Typical arenavirus particles were seen in platelet demarcation channels although there was no evidence of cytoplasmic changes or the formation of viral-induced inclusion bodies.

In parallel with human infections, maturation of Junin virus in tubular and epithelial cells of the kidney in guinea pigs seems to occur by budding intracytoplasmically into vesicles rather than at the plasma membrane (Cossio et al., 1977). Virus particles, morphologically indistinguishable from extracellular virus produced in other organs, were seen budding from the walls of the endoplasmic reticulum and there was a concomitant extension of the rough endoplasmic reticulum and convolution of the cisternae. These and similar observations have been readily supported by intensive immunofluorescent staining of infected cells paralleling the detection of virus structures by electron microscopy; however, this is not always the case in many tissues. Although the use of specific antibody may produce staining indicative of viral antigen present in large amounts, complete virus particles are not always discerned (Murphy et al., 1973). Several possibilities may account for this; either small samples might not contain involved sites of virus production or these observations may reflect an underlying mechanism controlling viral gene expression within different cell types. Murphy and colleagues suggested that this disparity might reflect variation in the capacity of cells to either accumulate viral

antigen, as detected serologically, or to produce the potentially infectious virus seen in the electron microscope. Such a difference may reflect ultimately the extent of direct cytopathology seen in different arenavirus infections *in vivo*. The study by Rodriguez et al. (1983) showed clearly that LCM virus infects specific populations of neurones in persistently infected mice; only nucleocapsid antigen would be detected with a lack of reactivity at the surface of the cells for viral envelope glycoprotein. This suggests differential synthesis of specific gene products within persistently infected cells which may lead to accumulation of viral antigen, in this instance in the absence of gross morphological changes and the production of mature virus particles.

Van der Zeijst et al. (1983a) have shown in BHK-21 cells persistently infected with LCM that intracellular ribonucleoprotein complexes were not infectious, frequently being associated with ribosomes and polysomes. Although there was no evidence of extracellular virus production, there was some evidence of intracytoplasmic vacuoles containing numerous LCM nucleocapsids which may arise by budding through the internal membranes constituting the endoplasmic reticulum or Golgi apparatus. Evidence for this was the finding that the infectious intracellular fraction was very large and heterogenous in size together with the sensitivity of infectivity to detergents. In addition, infectivity appeared resistant to both trypsin and RNase, suggesting the viral components necessary for infectivity were masked, presumably by a lipid bilayer. The rare occurrence of these particles as visualized by thin section microscopy precluded further studies, although the authors did point out that intracellular particles presumed to represent the infectious entities under study were considerably smaller in diameter than conventionally prepared LCM virions with a diameter of 50 nm and similar to the small particles reported by other workers studying thin sections of infected cells (Mannweiler and Lehmann-Grube, 1973; Maiztegui et al., 1975). Although such particles may be of importance in understanding viral morphogenesis in persistently infected cells, further studies are required as to the nature and biological function of these smaller particles.

6.2. Virus morphology

Electron microscopy of negatively stained preparations has shown that arenavirus particles are pleomorphic, enveloped structures with an average diameter in the range of 80 to 150 nm (Dalton et al., 1968; Mannweiler and Lehmann-Grube, 1973; Murphy and Whitfield, 1975), although

particles up to 500 nm are not uncommon (Pedersen, 1979). A careful study of Machupo virus size by Murphy et al. (1969) showed a skewed size distribution from 60 to 320 nm with a mode value of 110–120 nm. In this study, there appeared no significant difference in size distribution between virus grown *in vitro* and Machupo virus identified in the lymphoid organs of infected rodents.

The observed variation in size appears to be dependent on the time of harvest from infected cells, with larger particles being produced later in the infectious cycle. It has been suggested that as infection progresses larger areas of virally altered cell membrane provide a focus for the maturation of larger diameter virions concomitant with envelopment of progressively larger nucleocapsid aggregates (P.R.Young, personal communication). One consequence of this would be a decline in the particle to infectivity ratio later in infection whereby more genetic material becomes enveloped in one unit virus particle. A further important point is that particle size may be seriously underestimated by thin section analysis. It has been pointed out that preparations of infected cells containing either LCM (Mannweiler and Lehmann-Grube, 1973; Van der Zeijst et al., 1983a) and Junin virus (Maiztegui et al., 1975) may contain a second smaller population of particles, approximately 50 nm in diameter in the case of Junin virus. These particles appeared exclusively within cytoplasmic vacuoles having matured at the endoplasmic reticulum. However, an alternative explanation would be an apparent reduction in size estimation resulting from the thin section not including the centre of the virus particles observed in any one section. It is worth noting that at the other end of the size spectrum, many abnormally large and bizarre extracellular virus structures may result from the use of polyethylene glycol for the concentration of virus from tissue culture fluids (P.R. Young, personal communication).

The viral envelope, formed from the plasma membrane of the infected cell, contains surface projections 5–10 nm long which appear club-shaped with a hollow central axis when viewed end-on (Fig. 6.2), although one report does exist suggesting that the surface projections are artefacts of

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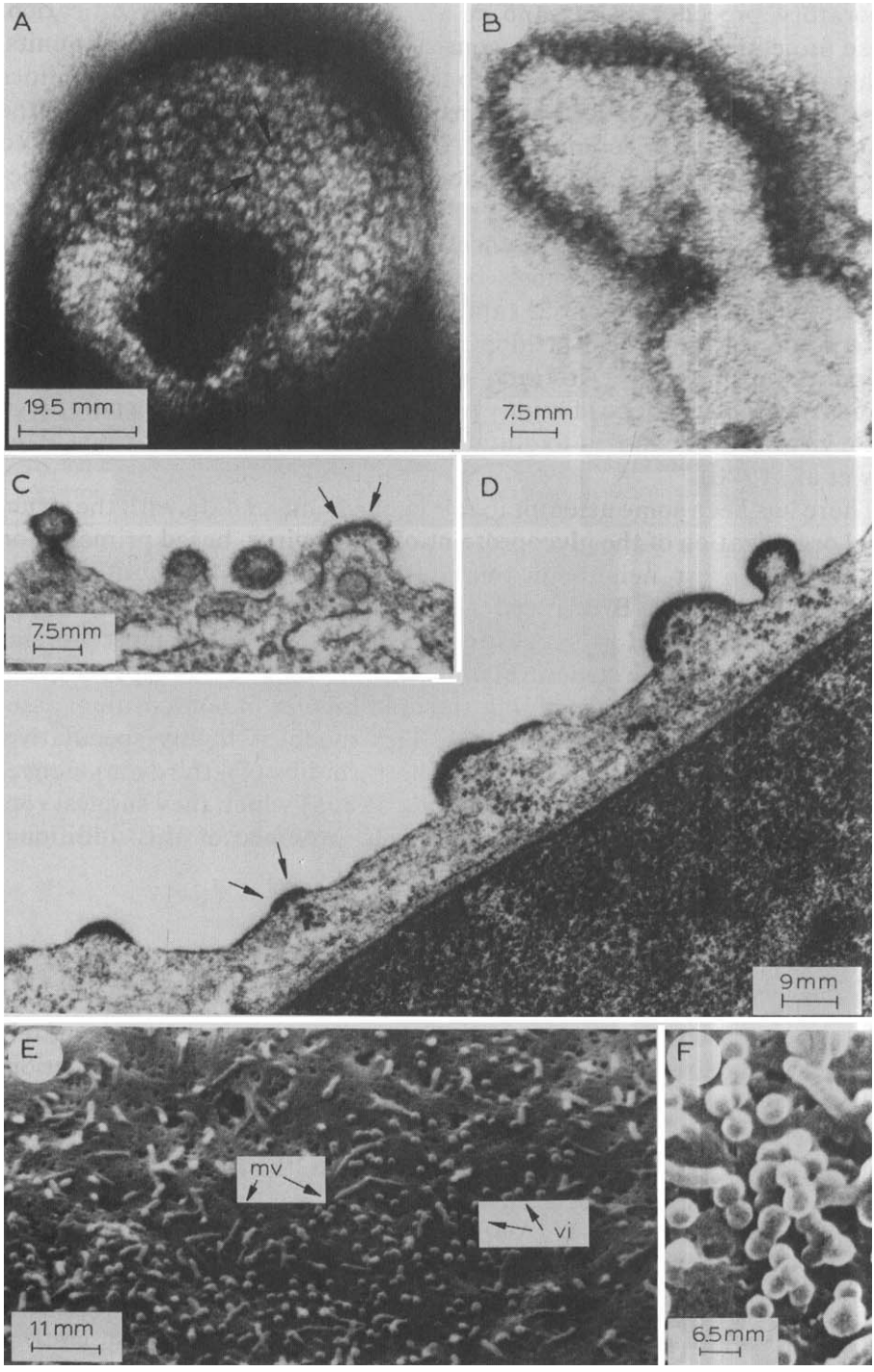
Fig. 6.2. Electron micrographs of Pichinde virus: (A, B) purified virus negatively stained with 2% PTA, pH 6.8 showing surface spike morphology; arrangement of the spikes in 5 and 6-fold symmetry can be seen (arrowed). (C, D) Ultrathin sections of infected Vero cells revealing the alignment of ribosomes beneath the membrane of budding viruses (arrowed in C) and discrete areas of membrane thickening associated with these viral buds (arrowed in D). (E, F) Scanning electron microscopy of infected Vero cells. A uniform population of budding viruses (vi) particles 110–130 nm in diameter can be seen amongst host cell microvilli (mv). Bars represent 50 nm in (A) and (B) 200 nm in (C), (D) and (F) and 1 μm in (E) (from Howard and Young, 1984).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B
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C
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D
9 mm
E
mv
vi
11 mm
F
6.5 mm

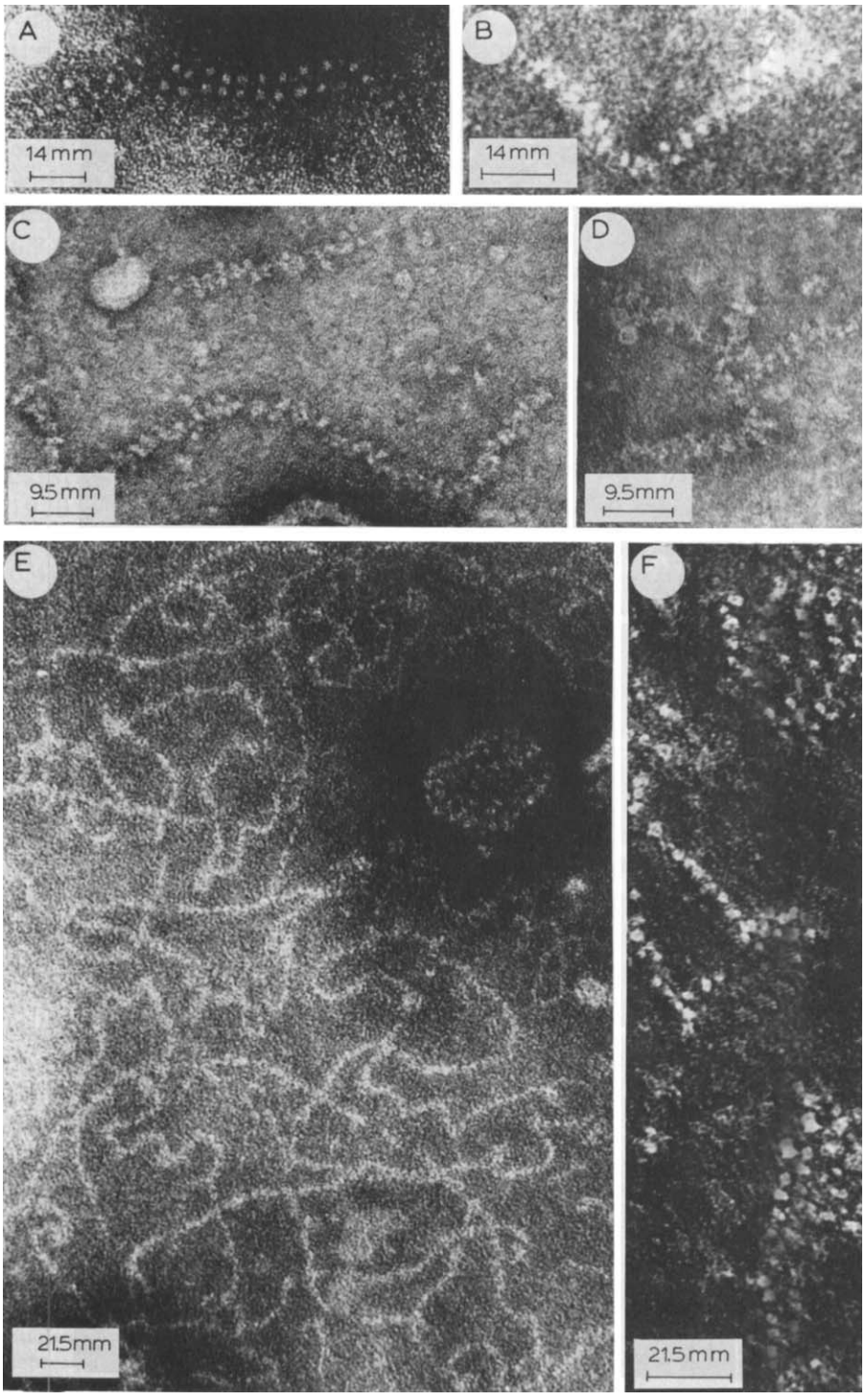
laboratory procedure (Lascano et al., 1979). At high magnification, these projections can be seen as consisting of discrete globular subunits, although no information exists as to the number or type of glycopeptides present in individual structures. Considerable variation is observed in the spacing of these projections, some virions containing a tightly packed lattice of surface spikes whereas other, larger particles show a more widely spaced random arrangement (Murphy et al., 1970; Young and Howard, 1983). These differences almost certainly reflect varying degrees of swelling by osmosis between different virus preparations. Although generally present as random arrays, particles with projections ordered in a symmetrical fashion may be seen in fresh, unfrozen virus preparations (Fig. 6.2). Attempts to examine by negative staining techniques the viral envelope of highly pathogenic arenaviruses are hampered if the virus is first exposed to glutaraldehyde or osmium tetroxide (Murphy et al., 1970).

There has been some attempt to relate biochemical data with the structural organization of the glycoproteins of LCM virus, based primarily on analyzing nearest neighbour relationships using a homobifunctional cross-linking agent (Bruns and Lehmann-Grube, 1983). The results have been interpreted as suggesting both major envelope glycoproteins are organized into one structural unit with the smaller being proximal to the membrane, possibly spanning the lipid bilayer in non-covalent association with the inner nucleocapsid. This model is highly speculative, however, and is complicated by the authors' finding of a third major envelope glycoprotein structure (Bruns et al., 1983a) which they suggest represents a discrete surface projection. The presence of this additional moiety has yet to be independently confirmed.

6.3. Internal structures

The internal architecture of extracellular virions cannot be adequately resolved by negative staining of either whole virus or by the thin sectioning of viral pellets. Muller et al. (1983) have suggested that dense core components separated by a clear translucent zone are a feature of infectious LCM virus particles but did not elucidate the fine structure of the

Fig. 6.3. Electron micrographs of (A–D) RNP-like structures released from virus particles disrupted by osmotic shock and negatively stained with 2% PTA pH 5.0 (E) linear arrays of nucleosomes released from purified Pichinde virus, negatively stained with 2% PTA pH 5.0; (F) nucleosome filaments in purified nucleocapsids isolated from detergent-disrupted virus by separation in a Urografin gradient and resuspended at low ionic strength (see text); negatively stained with 2% PTA pH 6.8. Bar markers represent 50 nm in (A–D) and (F), and 100 nm in (E) (from Young and Howard, 1983).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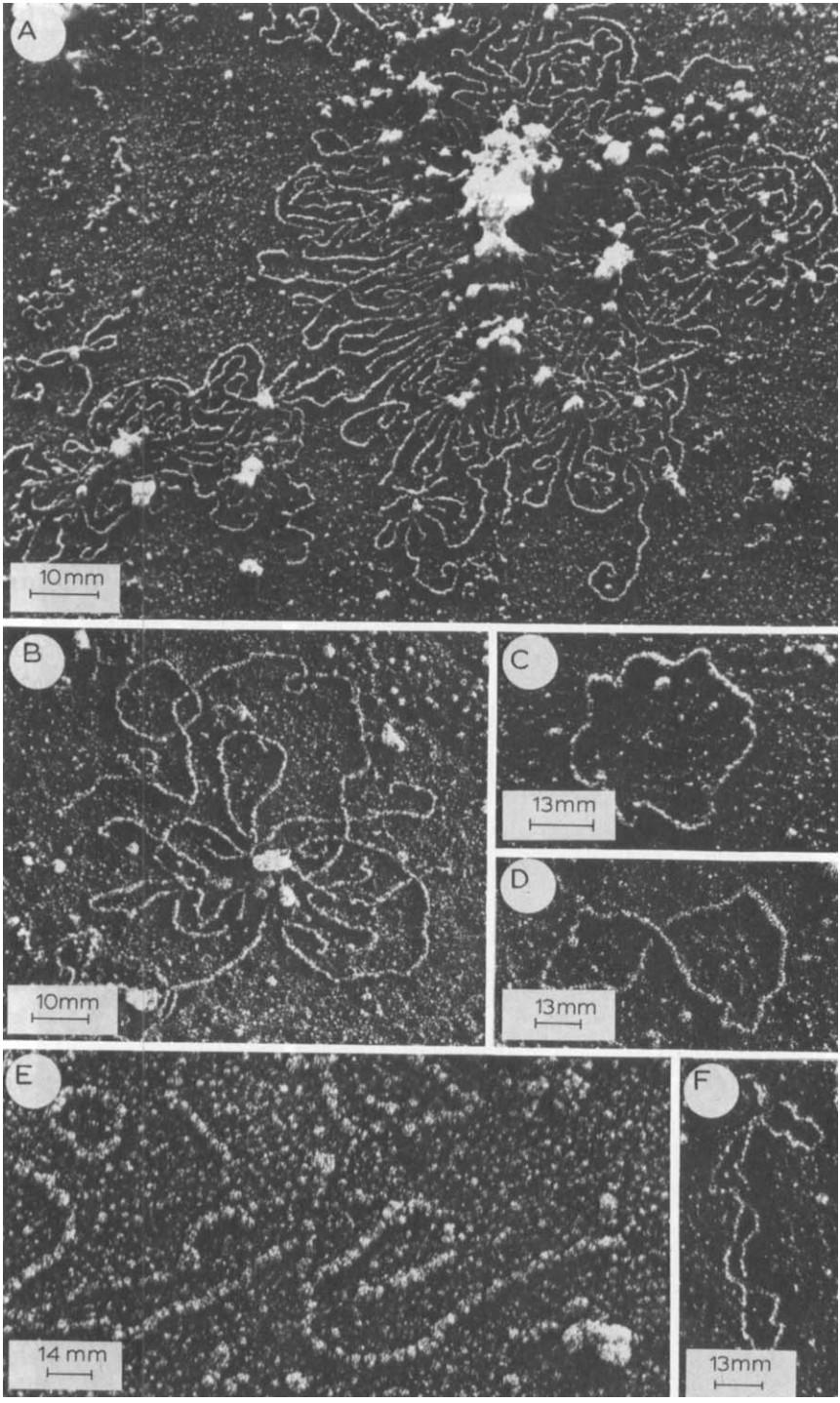
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virion nucleocapsids. This has only been achieved by prior disruption of the outer envelope. Isolated nucleocapsids of both Tacaribe and Pichinde viruses have been isolated free of contaminating host ribosomes by non-ionic detergent solubilization and density gradient centrifugation (Palmer et al., 1977; Vezza et al., 1977; Young and Howard, 1983). Strands with a 'beads-on-a-string' appearance that are 5–10 nm in diameter have been described representing a linear array of nucleosomal subunits (Fig. 6.3). The size of each structural unit is consistent with the presence of a single molecule of the major N polypeptide and differential organizational levels of this linear ribosome-like ribonucleoprotein structure have recently been reported (Young and Howard, 1983). Disruption of purified Pichinde virus by osmotic shock releases fibre-like structures 12 nm in diameter with occasional globular condensations which may reflect an enhanced association between neighbouring turns of an underlying helical structure, as has been described for cellular chromatin (Thoma et al., 1979). Rotary shadowing techniques of isolated ribonucleoprotein showed the spread of 15 nm strands from discrete core structures (Fig. 6.4) and when sufficiently dispersed the nucleoprotein appears as closed circles varying in length from 450 to 1300 nm; however, this variability appears to have no direct relationship between the two unique viral RNA segments. However, length distribution analysis of Tacaribe virus nucleocapsids showed a distribution into two distinct size classes of 640 nm and 1300 nm respectively (Palmer et al., 1977) and Pedersen and Koningshofer (1976) showed by rate zonal centrifugation of solubilized LCM virus that two distinct classes of nucleocapsid could be isolated. In the latter study, a heterogenous population with average size 123–148S containing both RNA species and a smaller 83S structure containing only S RNA were found. The frequent appearance in electron micrographs of pan-handled structures and the observation that denatured viral RNA is predominantly linear (Vezza et al., 1978a) would suggest that circular nucleocapsid forms may arise as a result of base pairing at the termini of RNA molecules, not dissimilar to the observation that complementation exists between the 3′ and 5′ terminal ends of RNA segments isolated from the bunyavirus Uukuniemi (Parker and Hewlett, 1981). Indeed, a recent report has shown that base pairing between either the L and/or S RNA genome strands may occur (Auperin et al., 1982a).

Fig. 6.4. Electron micrographs of purified nucleocapsids rotary shadowed with (A–E) gold-palladium or (F) platinumcarbon at an angle of 10°. (A) Large nucleocapsid aggregates; (B) 'Spider-forms'; (C–E) isolated circles in various stages of supercoiling; (F) nucleocapsid fibres as linear arrays of 15 nm beads (compare with Fig. 6.3). Bar markers represent 200 nm in (A) and 100 nm in (B–F) (from Young and Howard, 1983).

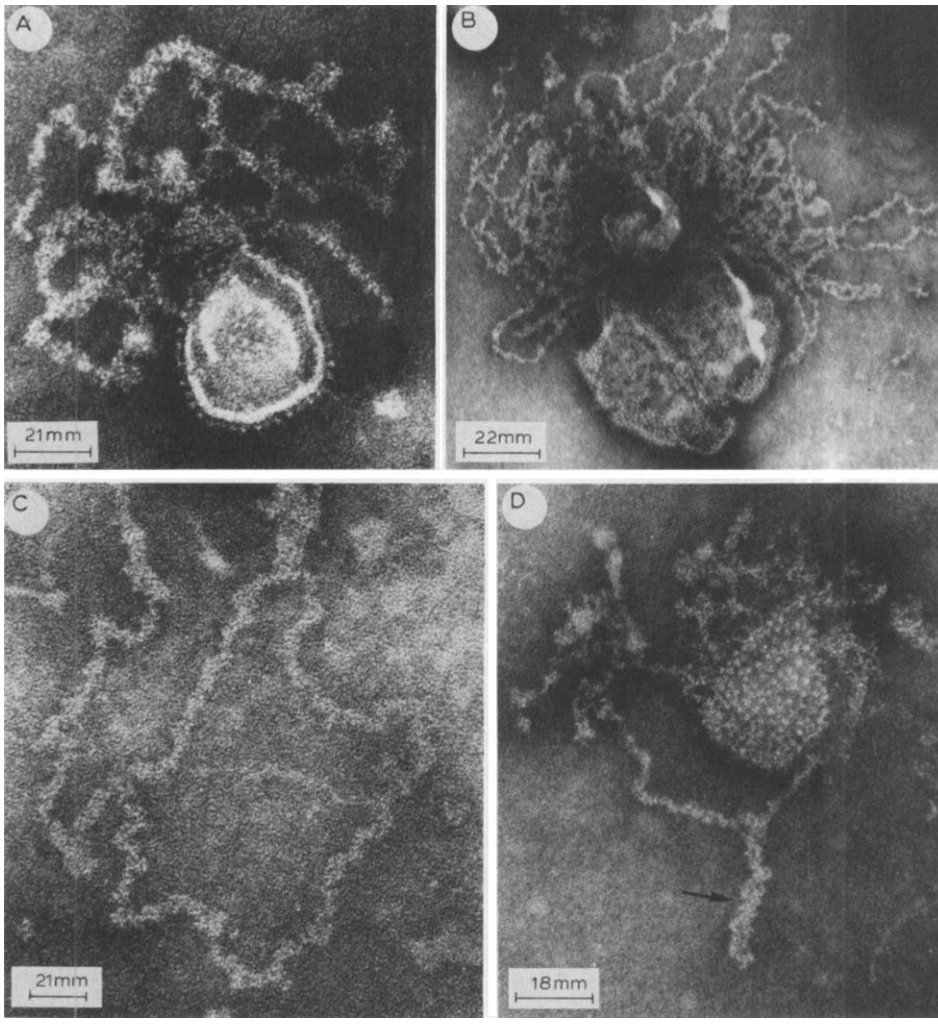

Fig. 6.5. Purified virus particles lysed by osmotic shock and negatively stained with 2% PTA pH 5.0 shows the release of 12–15 nm diameter fibres. Twisting and supercoiling of these fibres into thicker 20 nm strands is frequently observed (arrowed in A and D). Bar markers represent 100 nm in (A), (C) and (D) and 200 nm in (B).

The packaging of nucleocapsids during the process of virus assembly and maturation may evolve in degrees of supercoiling of circular ribonucleoprotein structures. This is supported by the finding of regularly sized 20 nm strands in virus cores and is compatible with the presence of supercoiled nucleocapsid fibres seen released from spontaneously disrupted

virions (Fig. 6.5). The core component thus appears to be organized as a result of the extensive convolution of these supercoiled fibres. Young and Howard (1983) have pointed out that variability in the amount of nucleocapsid material packaged into any one virus particle indicate that any specific control of this process may be limited. Variation in the specific arrangement of nucleosomes within each fibre of the core structures may reflect a variable number of nucleosomes per unit turn of the helix, with an increase in the number of nucleosomes per turn accompanied by a loss in resolution of individual subunits as they become more closely associated. The most stable helical configuration appears to be a 12 nm fibre structure regularly seen in spontaneously disrupted virus preparations (Fig. 6.5) and may contain between 6 and 8 nucleosomes per turn of the helix. Any further structural condensation results in little increase in the diameter of this structure. The extent of supercoiling appears to be directly related to ionic strength; as salt concentration is reduced, a progressive unfolding of higher order structures occurs, ultimately giving rise to the linear nucleosome array (P.R.Young, personal communication). This may be explained as a consequence of electrostatic repulsion between adjacent charges on the helically organized RNA which is progressively neutralized with increasing ionic strength.

At present it is unclear as to what role, if any, proteins other than the major nucleocapsid N protein may play in the architecture of the arenavirus nucleocapsid. Pichinde virus cores released by detergent treatment in the presence of 0.2 M NaCl contain traces of various polypeptides which may be removed by increasing the molarity of NaCl to 0.8 M NaCl. Under these conditions, only minor 72 000 and 15 000 molecular weight polypeptides remain associated with the virus nucleocapsids (Young et al., 1981). However, it is not clear if these represent tightly bound contaminants or virus-specific products with a functional role in either the assembly of the nucleocapsid and/or expression of the viral genome. The 72 000 molecular weight polypeptide is consistent with the finding of a similarly sized, 'P' protein found in nucleocapsid preparations of Tamiami and Tacaribe viruses (Gard et al., 1977; Vezza et al., 1978b). Although this protein was not found in immunoprecipitates of either infected cell extracts or purified virus preparations (Harnish et al., 1981), its specific association with arenavirus nucleocapsids and its appearance with purified virus grown in different host cells does suggest that it possesses some functional role in virus replication. A number of proteins which remain bound in low salt concentrations include a proportion of the total G2 glycoprotein component of the whole virus particle (Chapter 7). This may reflect a salt-dependent interaction between

nucleocapsid and envelope glycoprotein mediated by a specific ionic transmembrane association between the carboxyl-terminal end of the G2 polypeptide exposed on the inner face of the plasma membrane with the underlying nucleocapsid. Such electrostatic bonding has been demonstrated for Semliki Forest virus glycoprotein (Ziemiecki et al., 1980; Smith and Brown, 1977) and may be of importance in initiating virus assembly at cellular membranes.

The extent and nature of interaction between viral nucleocapsids and host ribosomes remains unclear. Both are clearly seen together in huge cytoplasmic aggregates (Abelson et al., 1969; Rodriguez et al., 1983) and a specific interaction between ribosomes and either the RNA or nucleocapsid protein would account for the appearance of ribosomes within nascent virus. The presence of host cell ribosomes within extracellular virus has been difficult to confirm on morphological grounds alone. However, core components of both LCM and Pichinde may readily be separated from the denser host ribosomes by gradient centrifugation in high salt (Pedersen and Koningshofer, 1976; Young and Howard, 1983). Farber and Rawls (1975) noted that an association between Pichinde virus nucleocapsids and ribosomal material is maintained following disruption in ionic conditions which would favour the continued higher order nucleocapsid structures and its interaction with the G2 envelope glycoprotein.

CHAPTER 7

Physicochemical properties and chemical composition

The biophysical and biochemical properties are consistent with the lipoprotein nature of the arenavirus particle. One or more virus-specific proteins located within the outer viral envelope bear the antigenic determinants responsible for eliciting host-immune responses; although the rapid development of monoclonal antibodies is increasing our knowledge of the serological properties of arenaviruses (see Chapter 3) much more requires to be known regarding the chemical composition, architecture and assembly of the extracellular virion. For example, the exact location of viral polypeptides responsible for both endogenous RNA polymerase and protein-kinase activities and the interactions between structural proteins located in the envelope and internal constituents remain to be defined. Additionally, it is as yet unclear as to exactly how persistence of virus is related to changes, if any, in particle structure, chemical composition or virus-specific modifications of viral proteins. Definitive studies as to the biophysical and biochemical properties are therefore a prerequisite for understanding arenavirus pathology and the virus-host relationship. These studies are hampered by the relatively poor growth yields of arenaviruses in tissue culture compared to many other viruses, making the application of recent methodology including the molecular gene cloning techniques currently in progress particularly exacting. However, there are few viral diseases which compare to LCM virus infection of the mouse where this knowledge could be applied to such an extensive body of information known about the influence and interactions between virus

and host-immune defence mechanisms. Other exciting problems which remain to be defined in molecular terms include the fine distinction between arenaviruses pathogenic and non-pathogenic for man and the unique close association between maturing virions and the functional ribosomes of the host.

7.1. Biophysical properties

The buoyant density of those arenaviruses so far characterized are approximately equal, reflecting the similar chemical composition of all members in the family (Table 7.1). Sedimentation values in the range of 300–350S have been calculated but the size variation within any one preparation of any particular arenavirus prevents an exact estimation of sedimentation coefficient. The increasing size of particles released late in cultures of infected cells (P.R. Young, personal communication) further complicates the preparation of virus for biophysical analysis. Particles up to 500 nm are not unusual (Pedersen, 1979). No information is available as to other biophysical parameters, e.g. diffusion coefficient and particle specific volume, although the molar composition of individual amino acids in LCM virions has been determined (Howard and Buchmeier, 1983).

TABLE 7.1.
Physicochemical properties of arenaviruses[a]

	Buoyant density, g/cm^3			S-value
	Sucrose	CsCl	Amido trizoates	
Old World[b]				
LCM	1.17	1.22	1.12–1.14	360 S
Lassa				
New World[c]				
Tacaribe	1.17	1.20		
Junin	1.17			
Machupo	1.17			
Pichinde	1.18	1.19–1.20		300–325 S
Tamiami	1.15			
Amapari	1.18			

[a] Data collected from Buchmeier et al. (1978), Gard et al. (1977), Gschwender et al. (1975), Mifune et al. (1971), Pedersen (1970, 1979), Rawls and Buchmeier (1975).

[b] No data available for Mopeia and Mobala viruses.

[c] No data available for Latino and Parana viruses.

The infectivity of arenaviruses is sensitive to changes in pH, temperature and certain salt ions. They may also vary in their stability to physical stresses, especially upon purification and/or storage.

Arenaviruses are very stable in the pH range 6.0–5.0 but infectivity declines rapidly at more acidic pH values. For example, a particular problem in the handling of Amapari-infected urine was reported by Pinheiro et al. (1977) who found that the titre of infectious virus fell by nearly 3 logs after standing for 12 h at ambient temperature, with a low pH accelerating loss of infectivity. Pichinde virus lost over 3 logs of infectivity when exposed to pH 3.4 for 2 h at room temperature (Mifune et al., 1971) and Machupo virus became almost completely inactivated at pH 5 and below (Webb et al., 1967). Lassa virus is similarly sensitive to low pH (Trofimov et al., 1981).

Investigations of RNA viruses have shown that thermal inactivation is generally complex occurring by several thermodynamically independent mechanisms. Loss of infectivity at lower temperatures is generally the result of nucleic acid denaturation, whereas at higher temperatures denaturation of structural protein components results in an accelerated loss

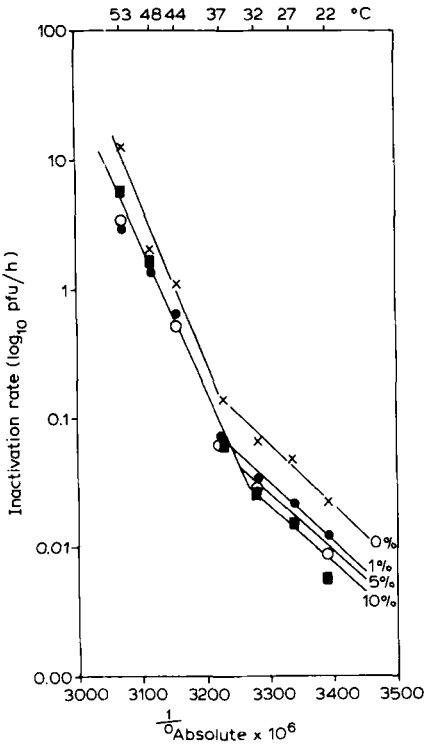

Fig. 7.1. Relationship between temperature and inactivation rate of Pichinde virus in medium containing increasing amounts of foetal calf serum (from Allison et al., 1985).

of infectivity (Dimmock, 1967; Fleming, 1971; Laude, 1981). In an early paper Mifune et al. (1971) estimated that Pichinde virus seemed to be relatively stable at lower temperatures: over 50% of infectivity remained after 3 days at 4°C with stability declining to 50% in 8 h at 25°C and 2 h at 37°C. Infectivity was lost rapidly at 56°C. A more detailed study has recently been reported (Allison et al., 1985). Inactivation of Pichinde virus occurred as a first-order reaction over the temperature range 8° to 37°C. The two mechanisms of inactivation became apparent by expressing rates of inactivation with respect to absolute temperature as an Arrhenius plot (Fig. 7.1). This showed that the two different inactivation reactions, represented by two different slopes, intersected at 37°C. At this temperature the half-life was estimated at 6.5 h as compared to 22 days at 8°C. The calculated energy of inactivation at low temperatures was found to be within the range established for low temperature inactivation of other RNA viruses where inactivation results from genome denaturation. Although the results were not analyzed in the same manner, Webb et al. (1967) found that Machupo virus infectivity was reduced more rapidly at 56°C and 37°C compared to virus held at 24°C and 4°C. However, visual examination of the results presented by Webb et al. suggest that the overall ratios of thermal inactivation are much higher with Machupo virus compared to Pichinde virus. As with all enveloped viruses, the stability may be increased by the addition of exogenous protein; this is also partially reflected in the finding that dilute virus is more labile (Pedersen, 1970; Pfau and Camyre, 1967; Rawls and Buchmeier, 1975).

Lloyd et al. (1982) have provided useful data on the thermal inactivation of Lassa virus in serum. Complete inactivation of 6 logs of virus occurred after 90-min exposure at 56°C. The authors have pointed out that the use of shorter times, e.g. 30 min is not advisable for the heat inactivation of clinical fluids, and that 60 min at 60°C is to be preferred for routine use. In contrast, there is little change of virus titre with time under similar conditions at ambient temperature. The simultaneous presence of serum protein would be expected to increase the thermal stability of arenaviruses (Allison et al., 1985). Trofimov et al. (1981) estimated the reduction of Lassa fever virus infectivity at similar temperatures to Lloyd et al., but in the absence of serum proteins. This study showed that infectivity was lost after only 20 min at 56°C, slightly faster than the inactivation of Machupo and Pichinde viruses included in the same study. Again, however, virus stability was maintained at ambient temperature with only a 0.3–0.7 $\log_{10}$ reduction in titre over a 6 h observation period. It should be stressed that the inactivation kinetics as reported by Lloyd et al. (1982) and Allison et al. (1985) indicated that prolonged exposure times at higher temperatures are required for significant inactivation of highly

dangerous pathogenic arenaviruses suspected as being present in blood or tissue specimens. In any event, measured inactivation rates are obtained within the limits of sensitivity of the infectivity assay employed and complete absence of infectivity cannot be guaranteed. More suitable measures include the use of chemical agents of inactivation, e.g. beta-propiolactone. For example, Lloyd et al. (1982) found that a 0.2% solution rapidly inactivated Lassa virus within 30 min at 37°C. At present, no comparable data exists for the use of formaldehyde specifically for the inactivation of arenaviruses. Gamma irradiation from a cobalt-60 source may provide an alternative method of inactivating Lassa virus (Elliott et al., 1982). Kinetics of inactivation followed first-order kinetics, as expected from previous findings of Pichinde virus inactivation using ultraviolet light (Carter et al., 1973a). This implies that only a single ionizing event is necessary to destroy infectivity. Elliott and colleagues also successfully inactivated Lassa with a 20-min exposure under an ultraviolet light lamp adjusted to deliver 2000 W/cm^2, but has suggested that gamma irradiation is preferable as the antigenicity of viral proteins remains intact at exposures greater than 10-fold in excess of that required to destroy residual virus infectivity. A further advantage was that the kinetics were not significantly affected when virus was suspended in whole serum. As with all methods of virus inactivation, great care is required to adequately monitor and control the procedure, especially when physical methods are adopted. Indeed Elliott et al. stress that despite the consistency of the irradiation procedure, samples should always be checked for residual infectivity after exposure in order to leave no doubt about the degree of inactivation.

There is some indication that different strains of individual arenaviruses may display varying thermal stability. For example, Camrye and Pfau (1968) found that on storage at 4°C the CA-1371 (Armstrong) strain of LCM was considerably more stable than the Traub and WCP strains. The half-life of the Armstrong strain may be estimated from these authors' data as approximately 48 h at 4°C. Although some virological studies have shown that the thermal stability of viruses may be increased in the presence of certain mono- and divalent cations (Wallis and Melnick, 1962; Wallis et al., 1962) it has been found that thermal inactivation of LCM is further accelerated in the presence of these ions (Pfau, 1965a).

The value of accurately assessing the thermal inactivation rates of arenaviruses should not be underestimated. Apart from the problem of the safe handling of potentially infectious clinical material, the transmission of many arenaviruses is thought to occur by the aerosol route and/or

contaminated soil and surfaces. The relative stability of those viruses so far examined therefore indicates that shed virus may survive for prolonged periods in the natural environment prior to re-infection of new susceptible hosts. Furthermore, the development of attenuated vaccine strains, notably against Junin virus, will require a thorough knowledge of virus stability if candidate strains are to be used with maximum effect.

7.2. Virus purification

An almost exhaustive array of methods have been adopted for the concentration and purification of arenaviruses. Precipitation of virus from large volumes of tissue-culture fluid often represents the initial step prior to further separation. The most commonly adopted approach is in the use of polyethylene glycol (PEG-6000) in the presence of 0.4 M NaCl. Ramos et al. (1972) reported that addition of PEG-6000 to a final strength of 6% results in 100-fold concentration of Pichinde virus after an overnight incubation, although the total recovery of infectivity was somewhat low, being less than 50%. Much shorter times of exposure to PEG, e.g. 1–2 h, have been found to be as equally effective in concentrating Pichinde virus from large quantities of fluid; recovery of infectious virus is also improved by addition of PEG as a concentrated solution. In this way, the total recovery of virus approaches 100%. Additionally, brief exposure to PEG avoids the formation of bizarre and irregularly shaped particles resulting from viral envelope fusion (P.R. Young, unpublished observations). Other methods of concentration include direct sedimentation of small volumes in the ultracentrifuge suitable for the concentration of radiolabelled virus and the use of saturated ammonium sulphate. The latter method requires particular care in ensuring the solution is first neutralized to avoid possible acidic inactivation of virus: 1 h at 0°C was found suitable for the concentration of LCM (Pedersen, 1970) and Gard et al. (1977) recovered approximately half of Tacaribe virus present in tissue fluid after addition of ammonium sulphate to 50% saturation for 30 min. However, simultaneous presence of calf serum above 1% concentration presents problems for the use of ammonium sulphate as excessive precipitation of serum protein occurs (Pedersen, 1973). Methanol has been used for the initial step in arenavirus purification (Pedersen, 1966; Coto et al., 1972; Welsh et al., 1976) but, as with the general use of alcohols for the purification of viruses temperature control is critical, maximum concentration of virus being achieved below 5°C. Other means of concentrating arenaviruses include the addition of lead acetate

(Gschwender et al., 1965) and the use of dextran sulphate-PEG phase separation (Martinez Segovia and Diaz, 1968). However, neither of the latter techniques have been widely adopted for the initial stage of arenavirus purification. Gangemi et al. (1977) successfully used a molecular filtration system with a retention capacity of molecules in excess of 10^6 molecular weight. Both Machupo and Tacaribe virus in separate experiments were concentrated up to 100-fold from large volumes of fluid with little or no loss of infectivity. Although the careful use of PEG represents the best approach for virus concentration, filtration is more likely to be of use in the handling of large volumes prior to the purification of candidate vaccine arenavirus strains, as the presence of minor amounts of PEG in materials for human use is unacceptable. Partial purification following initial concentration is most conveniently achieved by differential or discontinuous gradient centrifugation. An early study by Pfau (1965b) showed that aggregation of virus following resuspension of a virus pellet into a small volume could be overcome by the addition of 8.5% sucrose to the resuspending buffer. Pfau also found that fluorocarbon could then be used for further purification but thorough resuspension of the LCM virus pellet was essential for optimum results. More often the first stage of arenavirus purification consists of centrifuging concentrated virus through sucrose solution at a concentration of 10–20% v/v onto a cushion of sucrose at a concentration of 50% v/v or a value in excess of virus buoyant density (e.g., Pedersen, 1973; Ramos et al., 1972). Although much of the cellular debris is retarded in the upper layer a considerable amount of debris bands close to the virus at the interface between the sucrose solutions. The virus, visualized as an opalescent band by direct illumination, must therefore be aspirated with care and inevitably makes a second centrifugation step indispensable for obtaining purified virus. This may be accompanied by the use of linear sucrose gradients prepared in a manner that the gradient encompasses the predicted buoyant density value of the virus (Table 7.1). This produces virus of sufficient quality for analytical work, although removal of unwanted sucrose is desirable prior to storage.

Gradient media other than sucrose may be used. For example, amido trizoate solutions such as Renografin and Urografin produce isotonic solutions of lower viscosity (for a general review of the use of these and other newer centrifugation methods see Rickwood, 1978). Gschwender et al. (1975) first advocated amido trizoate gradients for the purification of LCM and these have subsequently been used routinely for purification of this virus. Metrizamide is not widely used for arenavirus purification, however, owing to the loss of virus infectivity in this medium. Because of

the lipoprotein nature of the arenavirus particle, self-forming gradients of dense salts, e.g. caesium chloride, are not required for effective arenavirus purification, although virus may be detected as banding at the expectedly higher density value of 1.22 g/cm^3 (see Table 7.1). In this context, there is one study which has assessed the relative stability of LCM in different salt media (Pfau, 1965a). The finding here was that the infectivity losses were considerable after centrifugation in gradients of either caesium chloride, rubidium chloride or potassium tartrate. Potassium tartrate at lower concentrations has been used with some success in combination with glycerol for the initial purification of Pichinde, Tamiami and Tacaribe viruses (Vezza et al., 1977; Gard et al., 1977) and for the effective purification of Lassa virus (Clegg and Lloyd, 1983). The latter study also made use of the addition of 0.2 M glycine to the preparation buffer, a modification previously introduced by Gschwender et al. (1975) for the purification of LCM virus and also for the purification of Pichinde (Young, 1985).

One problem in the final purification stages is of more than one band of virus material in a buoyant density gradient (Fig. 7.2). This heterogeneity is in addition to the heterogeneity due to size variations that result in broad virus bands recovered from a rate zonal centrifuge gradient (Pedersen, 1970). To some extent, variation in hydration states may account for minor differences in buoyant density. Alternatively, the presence of defective-interfering (DI) particles may give rise to a proportion of virions with lighter density; DI particles obtained from persistently infected cell cultures banded with a single peak 0.01 g/cm^3 lighter than standard virus in both Renografin and sucrose gradients (Welsh and Buchmeier, 1979). Similarly, Tacaribe virus from persistently infected cells was found to be of lower buoyant density (Gimenez and Compans, 1980). An interfering particle of LCM was separated from standard virus only with difficulty, at best a partial separation being achieved by centrifugation (Martinez Peralta et al., 1981). It is possible that certain cell cultures may have endogenous viral contaminants with similar physicochemical properties which may co-purify with arenavirus particles. For example, L cells are known to produce relatively significant quantities of retroviruses which have a similar buoyant density to arenaviruses (Faras and Erikson, 1969).

A further point is that the peak of infectious virus does not generally coincide with the peak of total virus material recovered from buoyant density gradients, maximum virus titre being restricted to the denser shoulder of separated virions (Pedersen, 1970; Ramos et al., 1972; Vezza et al., 1977).

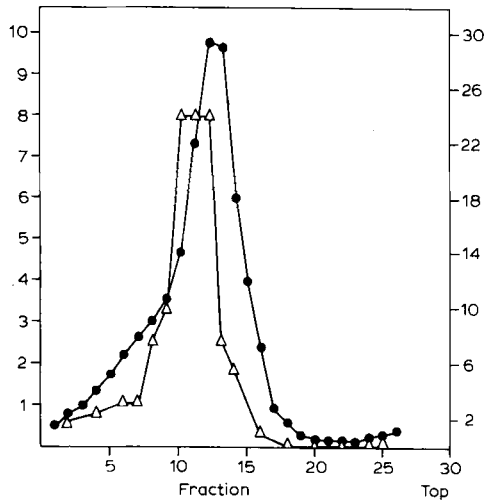

Fig.7.2. Rate zonal centrifugation of purified LCM virus in a 5–20% w/v sucrose gradient. Note the peak of radiolabelled virus is not coincident with the peak of virus infectivity; △–△, infectivity LD_{50}/0.30 ml x 10^{-5}; •–•, [^{3}H]uridine CPM x 10^{-2} (from Pedersen, 1970).

The internal nucleocapsids of both LCM and Pichinde viruses have been purified following solubilization of the outer viral envelope with a non-ionic detergent, e.g. Nonidet P40, Triton X-100. Ramos et al. (1972) subsequently banded nucleocapsid-containing material in linear caesium chloride gradients at a density of 1.32–1.34 g/cm^3 although some membrane-associated glycoprotein remained bound using low salt conditions. Vezza et al. (1977) alternatively separated viral nucleocapsids clean of contaminating envelope structures by solubilization using 2% Triton X-100 in the presence of 1 M NaCl, followed by centrifugation in caesium chloride; only nucleocapsid protein was recovered at a buoyant density of 1.31 g/cm^3. These authors also described the equal effectiveness of separating solubilized nucleocapsids by dextran sulphate-PEG phase separation, nucleocapsids free of envelope glycoproteins being recovered from the dextran phase. Alternatively, isolated nucleocapsids may be banded in amido trizoate gradients. Buchmeier et al. (1978) successfully recovered LCM nucleocapsids released by 2% Nonidet P40 in the presence of 1M KCl followed by banding at a density of 1.61–1.71 g/cm^3 in 20–50% w/v Metrizamide gradients. Untreated virus banded at the much lighter density of 1.12 g/cm^3. Unlike similar studies with Pichinde virus, high salt concentrations for effective solubilization did not appear to be critical for the removal of adhering envelope glycoprotein.

TABLE 7.2.
Size estimations of viral RNA in extracellular virions determined by gel electrophoresis

Virus	RNA Segment		Reference
	L	S	
Pichinde	2.63 - 2.83[a]	1.26 - 1.31[b]	Ramsingh et al. (1980)
LCM	2.58	1.3	Dutko et al. (1981)
Machupo	2.2	1.3	Lukashevich et al. (1984)
Lassa	2.2	1.3	Lukashevich et al. (1984)
Mobala	2.10	1.3	Gonzalez et al. (1984)

[a] Molecular weight x 10^{-6}.
[b] Value of 1.1 has been determined by direct sequencing (Auperin et al., 1984b).

In order to examine in detail the internal architecture of Pichinde virus, Young and Howard (1983) have examined several alternative procedures for the separation of nucleocapsid structures. Effective solubilization was achieved by incubation of purified virus with 1% Nonidet P40 in 0.5M NaCl for 30 min at 37°C, followed by isopycnic centrifugation in linear gradients of Urografin. Ribonucleoprotein was recovered at a buoyant density of 1.25 g/cm^3 and contained only trace quantities of residual envelope glycoprotein. The nucleocapsid fraction obtained by this method was seen by electron microscopy as strands of tightly packed beads 15 nm in diameter (see Chapter 6), in contrast to the helically organized filamentous structures seen by direct release of nucleocapsids resulting from osmotic shock. Alternatively, disruption of purified virus using a sonicator probe released core structures that could be separated by further centrifugation in a linear sucrose gradient. These core structures were found to have a buoyant density of 1.22 g/cm^3 with a size range of 80 to 120 nm in diameter (Fig. 7.2). These represented the interweaving of the 20–25 nm diameter strands of supercoiled fibres seen in nucleocapsids prepared by osmotic shock, in contrast to the condensed nucleoprotein subunit structures found in preparations obtained by detergent solubilization and subsequent centrifugation in Urografin. Whilst the latter method is preferable for the preparation of ribonucleoprotein suitable for biochemical studies, the integrity of the nucleocapsid architecture is dependent on salt concentration. The RNase sensitivity of the RNA genome in isolated nucleocapsid structures, however, warrants the use of the appropriate precautions against adventitious nuclease activity if such fractions are to be used as a source of purified viral RNA.

TABLE 7.3.
Protein composition of arenaviruses

Reference:	Ramos et al. (1972)	Vezza et al. (1977)	Gangemi et al. (1978)	Young et al. (1985)
(a) Polypeptides of Pichinde virus				
	72 000[a,b]	77 000	77 000	72 000
	72 000			
		66 000	68 000	62 000
		(N protein)	(N protein)	(N protein)
		64 000[b]	65 000[b]	54 000[b]
	34 000[b]	38 000[b]	38 000[b]	34 000[b]
				22 000
	12 000	12 000	15 000	15 000

Virus:	Tamiami	Tacaribe	Tacaribe	Machupo	Junin	Junin
Reference:	Gard et al. (1977)		Gangemi et al. (1978)		Martinez Segovia and de Mitri (1977)	Grau et al. (1981)
(b) Polypeptides of other Tacaribe complex arenaviruses						
					91 000	
	77 000	79 000	77 000	74 000	74 000	
	66 000 (N protein)	68 000 (N protein)	68 000 (N protein)	68 000 (N protein)	54 000 (N protein)	60 000
			50 000	50 000	52 000	44 000[b]
	44 000[b]	42 000	38 000	41 000	38 000	39–35 000[b]
			15 000	15 000	25 000	

Virus:	LCM	Lassa	Mopeia	Mobala
Reference:	Buchmeier et al. (1977)	Kiley et al. (1981)		Gonzalez et al. (1984b)
(c) Polypeptides of Old World arenaviruses				
			115 000	
		115 000	84 000[b]	
	63 000[a] (N protein)	60 000[c] (N protein)	60 000[c] (N protein)	60 000 (N protein)
	54 000[b]	52 000[b]	54 000[b]	48 000[b]
	35 000[b]	39 000[b]	40 000[b]	37 000

[a] Molecular weight.
[b] glycosylated.
[c] Revised values quoted in Gonzalez et al. (1984b).

7.3. Chemical composition

7.3.1. Proteins and glycoproteins

There is now a fairly common agreement that arenaviruses contain a major nucleocapsid-associated protein of molecular weight 54–68 000 together with one or two glycoproteins in the outer viral envelope (Table 7.3). Polypeptide analyses have been reported for all members of the Tacaribe complex except Flexal, Parana and Latino viruses; perhaps the most extensively characterized arenavirus is Pichinde among the New World arenaviruses and LCM virus as a representative of the Old World group. It should be noted, however, that although many isolates of Pichinde are available only the so-called 'prototype' (isolate no. 3739) and to a lesser extent the 'Munchique variant' (isolate no. 4763) have received attention. This probably reflects the comparative difficulty in obtaining yields of virus sufficient for biochemical analysis in all but a few selected isolates of this arenavirus. Similarly, attempts to characterize many arenaviruses have been hampered by the poor growth characteristics of the virus from original stocks.

The internal nucleocapsid-associated (N) protein accounts for much of the virus-specific protein present in purified preparations in either protein-stained polyacrylamide gels (Fig. 7.3) or analyses using metabolic radiolabels such as [^{3}H]leucine or [^{35}S]methionine. This protein remains bound to the RNA genome after solubilization of the virus with non-ionic detergents such as Nonidet P40 in salt concentrations up to 1.0 M. However, the genome remains susceptible to the action of RNase: nucleocapsids of both LCM and Tamiami viruses have been isolated but the RNA is greater than 90% susceptible to nuclease activity in this form (Pedersen and Koningshofer, 1976; Gard et al., 1977). It has been estimated that Pichinde virus contains over 1500 molecules of N proteins per virion, accounting for about 70% of the total protein composition (Buchmeier et al., 1977; Vezza et al., 1977, 1978b). Co-electrophoresis of arenavirus proteins shows that Pichinde and Tamiami N proteins are approximately the same size whereas the N protein of Tacaribe migrates slightly faster and that of LCM slightly more slowly. A similar comparison of LCM proteins and Old World viruses again has shown that LCM N protein migrates slightly slower when compared to the N polypeptides of Lassa and Mobala viruses (Gonzalez et al., 1984a). Given the close serological relatedness of many arenavirus N proteins, these slight but reproducible differences in electrophoretic migration may simply reflect slight differences in the proportion of charged amino acids rather than a true varia-

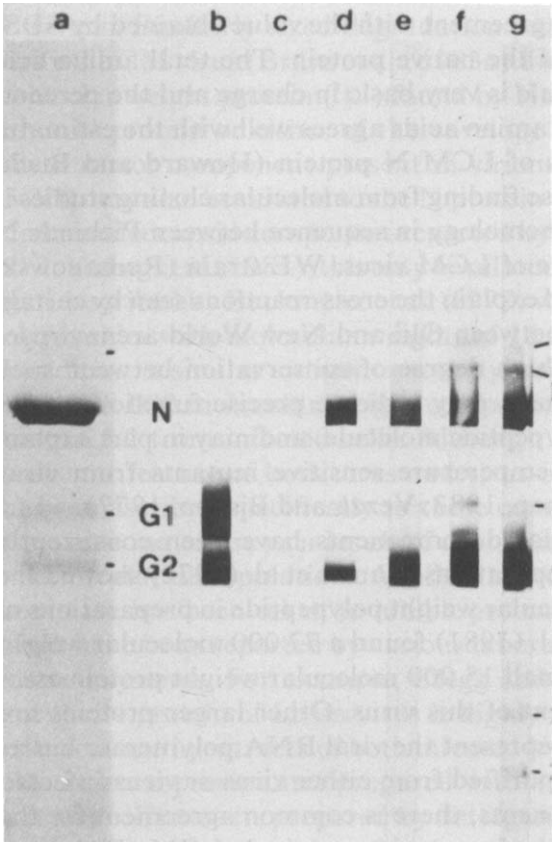

Fig. 7.3. Polypeptides of Lassa virus. SDS-gel electrophoresis of purified virions reveals the major nucleocapsid protein, N and the two envelope glycoproteins G1 and G2 (lane a). The envelope glycoproteins react with the lectin Concanavalin A (lane b). Reactions of individual proteins with either normal (lane c) or immune sera (lanes d–g) confirm the specificity of those components (from Clegg and Lloyd, 1983).

tion in molecular size. In support of this, it has been shown that the virion N proteins of Pichinde and the related Munchique variant possess minor differences as recorded by tryptic peptide digestion (Vezza et al., 1980; Harnish et al., 1983).

The N protein of LCM virus has been analyzed as being particularly rich in the charged amino acids aspartic and glutamic acids (Howard and Buchmeier, 1983), although a portion of these may exist as amines. Variable net charge among individual polypeptide chains may account for the wide heterogeneity in isoelectric point observed for this protein by Bruns et al. (1983a). The LCM virus N polypeptide also contains a high concentration of the hydrophobic amino acid leucine.

Auperin et al. (1984a) have recently identified the gene coding for the Pichinde N protein by direct nucleotide sequencing of cDNA made from viral RNA; this shows N is a 561 amino acid-long protein of molecular

weight 62 911 and is in good agreement with the value obtained by SDS-gel electrophoretic analysis of the native protein. The total amino acid composition shows the molecule is very basic in charge and the percentage composition of individual amino acids agrees well with the estimates obtained by direct hydrolysis of LCM N protein (Howard and Buchmeier, 1983). Indeed, a surprise finding from molecular cloning studies is the apparent 50% or greater homology in sequence between Pichinde N protein and the N polypeptide of LCM virus, WE strain (Romanowski and Bishop, 1984). This would explain the cross-reactions seen by certain monoclonal antibodies to N between Old and New World arenaviruses (Buchmeier et al., 1981). A high degree of conservation between such epidemiologically distinct viruses may indicate precise functional roles for certain areas of the N polypeptide molecule, and may in part explain the difficulty in recovering temperature sensitive mutants from virus stocks (Romanowski and Bishop, 1983; Vezza and Bishop, 1977).

Several minor non-glycosylated components have been consistently seen in purified arenavirus preparations. Ramos et al. (1972) showed the existence of a fourth, low molecular weight polypeptide in preparations of Pichinde virus and Young et al. (1981) found a 72 000 molecular weight polypeptide together with a small 15 000 molecular weight protein associated with the core component of this virus. Other larger proteins are frequently found which may represent the viral RNA polymerase but as yet this enzyme has not been purified from either virus or virus-infected cells. Among the other components, there is common agreement for the existence of a 20–25,000 molecular weight protein in LCM (Pedersen, 1973b), Junin (Martinez Segovia and de Mitri, 1977) and Pichinde virus (Young et al., 1985). It has been suggested by Pedersen (1979) that the 25 000 molecular weight protein of LCM virus may represent a matrix protein, although it was noted by Young et al. (1985) that the 22 000 molecular weight component labelled readily by several iodination procedures, suggesting that at least a portion of this protein is accessible at or near the virion surface. The relationship between these minor components is unclear. Peptide digestion experiments clearly show each of the major structural proteins of both LCM and Pichinde virus are distinct protein species (Buchmeier et al., 1978; Vezza et al., 1978). Harnish et al. (1981) have extended this work further by comparing tryptic digest patterns of virus-specific polypeptides immunoprecipitated from Pichinde-infected BHK-21 cells. At least six major proteins in the molecular weight range of 14 000 to 48 000 were resolved and all were found to contain peptides common to the intact viral N protein. Several of these (with molecular weights of 48 000 and 38 000) were found in purified virus and thus may represent proteolytic cleavage products of the major

N protein. Similarly sized degradation products have also been seen in purified LCM virus (Bruns et al., 1983a). Interestingly, the large 200 000 molecular weight protein ('L') found by Harnish et al. was not related to either N protein or either of the envelope glycoprotein structures. The fourth minor component present in virions with a molecular weight of 15 000 may also be related to the N protein being of equivalent size to the minor nucleocapsid component reported by other workers (Ramos et al., 1972; Young et al., 1981); this may be related to the complement-fixing antigen described by Buchmeier et al. (1977) which is composed of two small polypeptides with molecular weights of 15 000 and 20 000, respectively. In addition, a similar antigen reactivity was also found by Buchmeier et al. in a 28 000 molecular weight component isolated with an isoelectric point of 5.2 from infected cells. Although N-related proteins may be a consistent feature of both virus and virus-infected cells, no function has yet been ascribed specifically to these components; it is possible that at least some may arise as a result of contaminating protease action during experimental manipulations. Whilst this cannot be discounted entirely, great care has been taken to inhibit protease action yet N-related fragments have been reported by various laboratories studying other arenavirus-cell systems. For example, Clegg and Lloyd (1983) have reported that the N protein of Lassa virus is highly susceptible to limited proteolytic cleavage in extracts of both infected Vero and CV-1 monkey kidney cells. Immunoprecipitates prepared with a guinea pig polyclonal antiserum and cell extracts prepared by detergent lysis showed the presence of the prominent bands with molecular weights of 36 000 and 24 000, respectively. In addition to the sum total molecular weights of these fragments approximating the molecular weight of the intact Lassa virus N protein, there appeared an inverse relationship between their respective amounts; preparation of extracts under denaturing conditions inhibited the appearance of both the smaller bands in favour of the 60 000 molecular weight N protein. The extent of this proteolysis differed slightly, being more predominant in Vero cells. Notably, yields of many arenaviruses are poorer in Vero cells in contrast to other cell substrates. It is tempting to speculate that a reduced availability of N for packaging in these cells may be one feature leading to a correspondingly reduced amount of infectious virus.

Further evidence as to the specificity of N protein proteolysis comes from a recent comparison of immunoprecipitates made using cells infected either with the 3739 prototype Pichinde virus or its Munchique variant (Harnish et al., 1983). Both the N protein of the Munchique virus and its 38 000 molecular weight derivative migrated slightly faster in acrylamide gels and a second N-derived 28 000 molecular weight protein

was less in evidence. These observations suggest small but distinct phenotypic differences manifested by susceptibility to host-cell dependent protease activity; a recombinant virus containing the N protein of the Munchique variant also exhibited the 38 000 molecular weight N fragment with a correspondingly faster electrophoretic mobility. However, it remains to be determined as to the functional significance, if any, of such mechanisms in the arenavirus replication cycle. In the case of Lassa virus, the protease responsible for N protein cleavage seems remarkably resistant to inhibition by a number of widely used protease inhibitors (Clegg and Lloyd, 1983). Compans et al. (1981) found that gel electrophoresis of Tacaribe viral polypeptides with repeated changes of electrode buffer resulted in a dichotomy of N polypeptide material. Limited proteolysis showed both bands contained essentially similar amino-acid sequences, although it was unclear whether these differences represented minor cleavage of the N protein or other secondary modifications, e.g. phosphorylation. No significant amounts of either sulphate or phosphate have been found associated with arenavirus structural proteins (Vezza et al., 1977; Gard et al., 1977). However, extracellular LCM virus and Pichinde virus particles contain a protein kinase which phosphorylates the N protein in the presence of a suitable phosphate donor, e.g. ATP (Howard and Buchmeier, 1983; C.R. Howard, unpublished observations). This activity was found to remain associated with isolated nucleocapsids after solubilization of the viral envelope with Nonidet P40 and is not stimulated by the addition of cyclic nucleotides. Although these studies do not eliminate the possibility that the enzyme is a host enzyme non-specifically trapped within maturing virus particles, it is conceivable that phosphorylation may play a role early during infection. For example, it has been suggested that phosphorylation of vesicular stomatitis virus proteins may aid in the activation of the virion RNA polymerase and/or modify the enzyme template within the nucleocapsid prior to transcription (Witt and Summers, 1980).

The identity of the arenavirus-specific RNA polymerase remains unknown. However, Harnisch et al. (1981) have shown the existence of an 'L' protein in Pichinde virus with a molecular weight of approximately 200 000 which is unrelated to either N or GPC polypeptides, the latter representing the precursor molecule of the viral envelope glycoproteins. The small amount of this component together with the time kinetics of its appearance within infected cells is compatible with this hypothesis but direct evidence based on isolation of active RNA plymerase from this or any other arenavirus is lacking. A minor protein with a molecular weight of 72 000–79 000 has been found in many arenaviruses, and some workers have somewhat prematurely identified this as a candidate polymerase

('P') protein (Gard et al., 1977; Vezza et al., 1978b). Although in several instances these may represent virus-specific proteins closely associated with viral nucleocapsids (Young et al., 1981), Harnish et al. (1981) have pointed out that a 77 000 molecular weight host component can be found in Pichinde virions which may result in poor definition of viral proteins in this molecular size range. Furthermore, the Tacaribe 'P' and N proteins appear to have similar isoelectric points and at least some amino-acid sequences in common (Compans et al., 1981).

It is generally accepted that the major surface glycoproteins of arenaviruses are not primary gene products; Buchmeier and Oldstone (1979) have shown by peptide analysis and pulse-chase experiments that the two LCM virus proteins arise by proteolytic cleavage of the 75 000 molecular weight GPC glycoprotein present in immunoprecipitates of infected cells. In common with the cell-associated glycoprotein reported by Saleh et al. (1979) as present in Tacaribe infected cells, the LCM virion precursor glycopeptide was comparatively rich in mannose and glucosamine. In contrast, the larger 44 000 molecular weight glycoprotein present in mature LCM virus particles contains glucosamine, fucose and galactose, resembling the branched A-type glycosylation pattern as described by Johnson and Clamp (1971). These findings, together with the observation that the 54 000 molecular weight glycoprotein of Pichinde virus may be specifically radiolabelled after exposure to galactose oxidase (P.R. Young and J. Skelly, unpublished observations), suggests that shortening of at least some mannose-rich carbohydrate side chains of a precursor may occur followed by the addition of sugars such as fucose or galactose prior to cleavage. The extent of glycosylation remaining on the larger G1 glycoprotein may be variable, at least in the case of Junin virus (Grau et al., 1981). Nucleotide sequence studies have shown that a presumptive GPC gene may code for a protein rich in potential asparagine-linked glycosylation sites (Auperin et al., 1984b). This predicted gene product would be 503 amino acids in length and possess a molecular weight of 57 300. Harnish et al. (1981) found the 79 000 molecular weight GPC component of Pichinde virus in polyacrylamide gels migrated with a lower molecular weight of 42 000 when grown in the presence of the glycosylation inhibitor tunicamycin. Maturation and release of virus does not seem to be markedly inhibited in the presence of tunicamycin although glycoprotein processing appears to be essential for infectivity (Padula and Martinez Segovia, 1984).

In a preliminary report, Kiley et al. (1981) suggested that the high molecular weight glycoproteins present in Lassa and Mozambique viruses may represent similar viral glycoprotein precursor structures present in trace quantities and Clegg and Lloyd (1983) have found a

glycosylated 72 000 molecular weight protein in both purified Lassa virus and infected cells which reacts with specific antisera.

Somewhat at variance with the above observation is the suggestion by one group that another major 85 000 molecular weight glycoprotein is present in mature LCM virions (Martinez Peralta et al., 1981; Bruns et al., 1983a). As this band produced limited digest peptides of a size seen in digests of similarly treated GP1 and CP2 glycoproteins, the possibility arises that minor quantities of the precursor polypeptide are present in extracellular virus under the conditions adopted by this laboratory.

A common major glycoprotein species ('G2') in the molecular weight range of 34–42 000 has been found in all arenaviruses so far examined. This protein can easily be extracted from the outer viral envelope by non-ionic detergent treatment in conditions of low salt and approximately 300–400 molecules account for 10–15% of total protein present in Pichinde virions (Buchmeier et al., 1977; Vezza et al., 1977). The other major glycoprotein ('G1') is also variable in size although it is solubilized only under high salt conditions in the presence of non-ionic detergent. However, both glycoproteins are located on the virus surface as confirmed by two separate lines of evidence. Firstly, treatment of either LCM or Pichinde viruses with proteases specifically removes the surface projections resulting in the visualization of smooth enveloped particles by negative staining electron microscopy (Vezza et al., 1977; Buchmeier et al., 1978). A concomitant reduction in the presence of both glycoprotein species is seen by polyacrylamide gel electrophoresis. Secondly, both glycoproteins can be identified by the introduction of [^{125}]iodine using established surface labelling techniques (S. Sengupta and W.E. Rawls, unpublished observations, quoted in Rawls and Leung, 1979). Earlier studies suggested that G2 is the most exposed component of the Pichinde virus envelope (Ramos et al., 1972; Vezza et al., 1978); however, it is unclear from these studies whether the two envelope components definitely exist as discrete structural components or whether indeed the outer surface projections seen under the electron microscope consist of a homogenous population of structures composed of both G1 and G2 glycoproteins (see Chapter 6). Certainly the molecular ratio of both glycoproteins is approximately one (Buchmeier et al., 1977; Vezza et al., 1977). However, Young et al. (1981) found that antiserum specific for the G2 protein of Pichinde virus retained G2 molecules when an infected cell extract was passed through an affinity column, indicating that each glycoprotein may form separate structures in the viral membrane. Recently, Bruns et al. (1983b) have succeeded in isolating in pure form the larger G1 envelope of glycoprotein of LCM virus by affinity chromatography using a monoclonal antibody which neutralizes virus infectivity at high dilution. The

bound glycoprotein was readily recovered at low pH and subsequently found to inhibit virus neutralization by antibody showing that exposure to acid does not irreversibly alter at least some of the biological functions of this protein. This study again illustrates that there seems to be no covalent linkage between the envelope glycoproteins of LCM virus.

The puzzling feature of arenavirus structure is the apparent finding of a single glycoprotein in the envelope of Tacaribe and Tamiami viruses (Gard et al., 1977). This is despite the close relationship that is known to exist between Tacaribe and Junin virus, the latter having been characterized as possessing the more typical duplex of surface glycoproteins (Table 7.3). In order to exclude the possibility that Tacaribe virus contained two closely migrating or similarly sized envelope glycoproteins, Boersma et al. (1982) resolved Tacaribe virus glycopeptides using two-dimensional gel electrophoresis. Although as many as 10 distinct species of glycoprotein were separated in the second, isoelectric focussing dimension, all gave similarly sized protease cleavage products, thus indicating that Tacaribe virus indeed does contain a single glycoprotein in this molecular weight range. However, differences in glycosylation may account for the observed extensive variation of isoelectric point, particularly the content of sialic acid (Boersma et al., 1982). A comparison by pronase digestion of the G polypeptide from whole virus with the GPC structure found in infected cells showed that glycopeptides of the cell-associated 70 000 molecular weight GPC protein differed both in size distribution and the content of sialic acid. In particular, the GPC polypeptide contained mostly mannose-rich peptides in the molecular weight range of 1600 to 1900 after pronase digestion. In contrast, the cleaved G protein generated larger peptides of average size 3300 molecular weight rich in glucosamine and with terminal sialic acid. This study provides further evidence that the envelope protein(s) of arenaviruses are glycosylated initially by the addition of simple, mannose-rich oligosaccharide chains which are subsequently processed into branched complex moieties during viral maturation. A similar heterogeneity of both LCM virus glycoproteins by two-dimensional gel electrophoresis has been reported by Bruns et al. (1983a), suggesting that this mechanism of glycosylation may be general to all the arenaviruses.

It is not clear how the proteins of defective interfering arenavirus particles may differ, if at all, from standard infectious virus, although recent work indicates that the mRNA species coding for N and the glycoprotein GPC precursor could be independently regulated in persistently infected cultures (Auperin et al., 1984b). Welsh and Buchmeier (1979) analyzed defective interfering LCM virus derived from the CA-1371 (Armstrong) strain of LCM. Virus purified from persistently infected BHK-21 cells

passaged twice weekly for at least one year showed no difference in protein composition compared to standard virus, as detected by single dimension gel electrophoresis. Gimenez and Compans (1980) similarly analyzed defective interfering Tacaribe virus particles and found no qualitative difference in protein composition as compared to standard virus. However, virus purified from persistently infected BHK-21 cells did show a slight reduction in the electrophoretic migration rate of the major nucleocapsid (N) protein. There was also a decrease in the amount of the viral envelope glycoprotein in the same preparation, although this may simply reflect the smaller size of defective interfering particles, estimated at 55 nm diameter in contrast to a diameter of 95 nm for the standard virus. These results suggesting qualitative similarities between protein profiles of defective interfering virus and standard preparations are paralleled by findings with other persistent virus infection model systems (Huang and Baltimore, 1977). However, these findings are contrasted somewhat by the report of Martinez Peralta et al. (1981) who found significant differences in glycoprotein composition of a purified LCM virus preparation enriched for an interfering particle. These virions were believed not to be defective in the sense of requiring infectious virus for replication and contain diminished amounts of both G1 and G2 major LCM virus glycoproteins. Instead, a new glycoprotein of molecular weight 65 000 was found. It remains to be clarified as to the relationship between this polypeptide and the smaller G1 and G2 glycoproteins present in infectious virions, and indeed how these particles are related to the defective interfering particles characterized by other groups.

7.3.2. Nucleic acid

All evidence obtained so far indicates that purified arenavirus preparations contain at least two discrete pieces of single-stranded RNA of different sizes (Table 7.2). Pedersen (1970) showed that two major and one minor RNase-sensitive components were released from LCM virus by treatment with 1% SDS and could be separated in high-salt sucrose gradients into three populations of 28 S and 22 S and 18 S, respectively. LCM viral RNA was resolved into four fractions by PAGE, two of which comigrated with 28 S and 18 S cellular ribosomal RNA (Pedersen, 1971). The level of radiolabel incorporated into these two viral RNA species was markedly reduced by the addition of 0.15 μg/ml actinomycin D to the cell cultures at the time of infection; the yield of infectious virus was not significantly reduced under these experimental conditions. The remaining components were deemed to be virus-specific and estimated as having

S-values of 31 and 21, respectively. Pedersen and Koningshofer (1976) subsequently reported both RNA species were associated with a heterogenous population of ribonucleoprotein of average size 123–148 S. In addition, a second 83 S structure was found which only contained the 23 S viral RNA. The multicomponent nature of the arenavirus genome has since been confirmed for other arenaviruses including Pichinde, Junin, Tacaribe, Tamiami, Lassa and Machupo viruses. The total molecular weight of the arenavirus genome is variously estimated to be in the range of 3.2 to 4.8 x 10^6. Considerable secondary structure is known to be a feature of viral RNA (Rawls and Leung, 1979) thereby requiring the performance of size estimation studies under denaturation conditions. Ramsingh et al. (1980) in a careful study analyzed Pichinde viral RNA in a variety of gel media after denaturation by treatment with either methylmercury hydroxide or dimethyl-sulphoxide-glyoxal. The average molecular weights of the large (L) and small (S) RNA segments were determined as being in the ranges 2.83 x 10^6 and 1.26–1.31 x 10^6, respectively.

Direct sequence studies of Pichinde S RNA have shown the size of the S species to be exactly 3419 nucleotides long with a molecular weight of 1.1 x 10^6 (Auperin et al., 1984b). Assuming all of the viral genome is expressed as virus protein during infection, the total protein molecular weight coding capacity may be estimated at 250 000–300 000 from the L RNA and 125 000–150 000 from the S RNA. Similarly, Dutko et al. (1981) estimated the molecular weight of the L and S RNA species of LCM virus at 2.58 x 10^6 and 1.3 x 10^6, respectively. Comparative gel electrophoresis of viral RNA extracted from different arenaviruses shows some minor differences in relative electrophoretic mobility. For example, Gimenez and Compans (1981) showed that the S RNA species Tacaribe virus migrated ahead of that obtained from Pichinde. Although this may represent a minor difference in molecular weight such variations are likely to result from differences in RNA nucleotide sequences.

In most analyses using gel electrophoresis, the ratio of radiolabel incorporated into L and S RNA species is approximately one. If the rate of synthesis of each species is equal, this implies that there are approximately two molecules of S RNA for every one of L RNA (Pedersen, 1979). It is unclear whether this applies to individual virus particles; however, early ultraviolet light inactivation studies indicated that each virion contained only a single functional genome (Carter et al., 1973b). Although the incorporation of ribosomes into Pichinde virus particles suggests the possible presence of virus-specific RNA with positive polarity, all the evidence obtained so far confirms that arenavirus RNA does

not possess the structural properties typical of eukaryotic mRNA. Leung et al. (1977) found that Pichinde virus RNA did not contain any significant tracts of polyadenylic acid either before or after nuclease digestion; in addition, no evidence of either 5′-capping or methylated bases could be found. The failure of the same material to stimulate the synthesis of identifiable virus-specific polypeptides when added to the wheat-germ *in vitro* protein synthesizing system provided further evidence as the absence of messenger function. Hitherto extensive confirmation of these observations for arenaviruses other than Pichinde is lacking.

Further evidence as to the negative polarity of the arenavirus genome is the presence of a RNA-dependent RNA polymerase in preparations of Pichinde virus (Carter et al., 1974; Leung et al., 1979). All attempts to demonstrate reverse transcriptase activity have proved negative (Scolnick et al., 1970; Carter et al., 1974; Welsh et al., 1975).

Considerable evidence is now available to show that the electron-dense 20–25 nm particles visible within purified virions represent host cell ribosomes and that the 28 S and 18 S RNA extracted from Pichinde virus both possess a high guanosine plus cytosine (G + C) content and the methylated bases characteristic of ribosomal RNA. This study was complemented by the direct demonstration of ribosomal RNA associated with host ribosomes released from virus grown in BHK-21 cells prelabelled for 48 h with [^{3}H]uridine. These structures possessed similar physicochemical properties to ribosomes from uninfected BHK-21 cells (Farber and Rawls, 1975), and further experiments showed that, like cell ribosomes, 80 S structures extracted from Pichinde virus were susceptible to EDTA dissociation and contained both host-derived 28 S and 18 S RNA. Oligonucleotide analysis has shown conclusively that the 28 S and 18 S RNA structures of Pichinde virus are identical to those of non-infected host cell ribosome RNA (Vezza et al., 1978a). Heterogenous low molecular weight RNA has frequently been found within arenavirus particles and almost certainly represents further ribosomal RNA material (Carter et al., 1973; Pedersen, 1973a).

It should be noted that the total amount of ribosomal RNA and the relative proportion of its two species varies considerably (Vezza et al., 1977; Leung et al., 1979). Small amounts of 28 S RNA only were present in Pichinde virus grown from a freshly cloned stock of Pichinde virus previously passaged at 37°C. (Vezza et al., 1978a). However, the frequent lower ratio of 18–28 S RNA present in purified virus compared to uninfected cell ribosomes may reflect the higher frequency of 18 S RNA degradation during phenol extraction. The overall ribosomal RNA content may in turn be influenced by the varying proportions of infectious to

non-infectious particles present in virus stocks, although virus replication neither requires newly synthesized ribosomal RNA nor functional ribosomes (Carter et al., 1973a; Leung and Rawls, 1977). However, ribosomes extracted from Pichinde virions do have the potential to participate in *de novo* protein synthesis, but only after the addition of exogenous messenger RNA (Chinault et al., 1981).

Analysis of oligonucleotides obtained after T_1 enzyme digestion of the large and small virus-specific RNA components of arenaviruses has shown that each species is unique. A genetic analysis was undertaken by

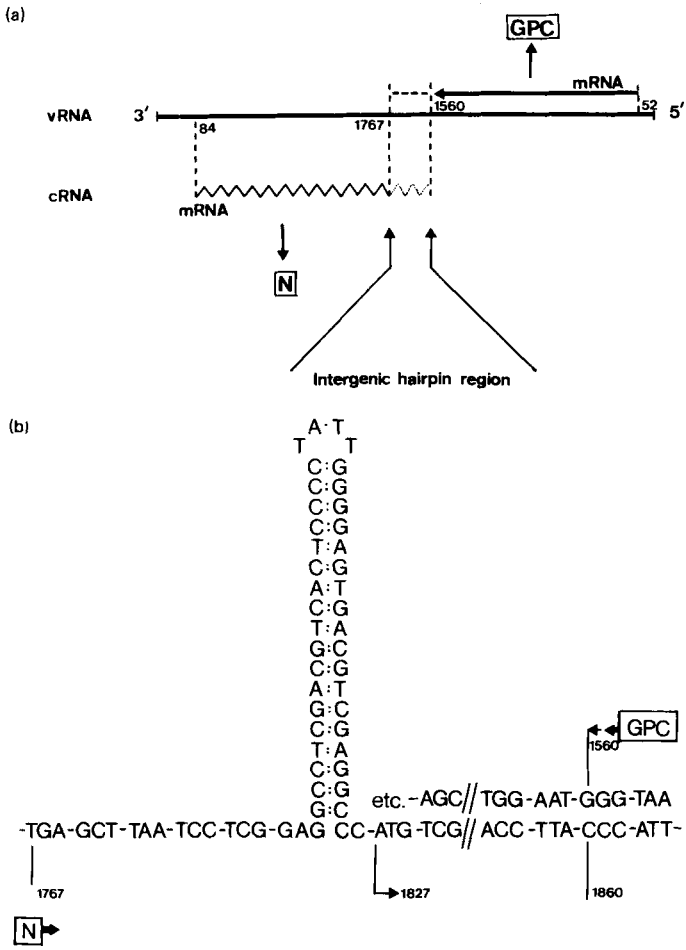

Fig. 7.4. Organization of the arenavirus genome. (a) Coding strategy showing the N protein is a translation product of mRNA transcribed from the negative-sense RNA genome. The envelope glycoprotein precursor, GPC, is conversely the product of mRNA with a coding sequence identical to the 5' end of the viral genome. (b) Detail of the 'intergenic' region of the Pichinde genome showing possible base pairing to form a hairpin structure. Note, however, these sequences may code for additional amino acids which could be expressed by suppression of the terminator codon of the N gene. Sequence information from Auperin et al. (1984a).

Vezza and Bishop (1977) in order to ascertain whether the two viral RNA species of Pichinde virus contained different gene sequences. The original 3739 prototype isolate of Pichinde virus does not replicate well at an elevated temperature of 39°C in either BHK-21 or Vero cells, the efficiency of plating compared to growth at 35°C being less than 0.01. These authors increased the yield of virus by consecutive passage at 39.8°C in BHK-21 cells prior to obtaining conditionally lethal temperature-sensitive (ts) mutants by exposure to 5-fluorouracil. All nine ts mutants obtained were plated with an efficiency ratio less than 0.0005 at 39.8°C with a corresponding 3 log drop in virus yield at the higher temperature. Dual infection of BHK-21 cells enabled the authors to classify these ts mutants into two non-overlapping genetic complementation groups of five and four mutants respectively. These results indicate that high frequency recombination, typical of viruses with a segmented genome, may occur among the arenaviruses. A third approach using cDNA made from L or S RNA templates as probes for nucleotide homology between Pichinde genome segments has also confirmed that both species contain predominantly unique sequences (Leung et al., 1981). Tacaribe L and S viral RNA similarly have been found to have unique sequences (Gimenez and Compans, 1981). Although there is a strong serological relationship between Tacaribe and Pichinde viruses (Chapter 3), the oligonucleotide patterns of both L and S Tacaribe RNA species are distinct from those of the corresponding material extracted from Pichinde virus.

Preliminary analysis has shown that extracted viral RNA is up to 20% resistant to RNase activity (Vezza et al., 1978a; Lukashevich et al., 1984). It is unclear at present whether this represents hybridization of complementary RNA sequences within the same or different RNA strands, although some degree of hairpin structure within the S strand has been predicted from nucleotide sequencing studies (Auperin et al., 1984a; Fig. 4b). Although circular nucleocapsid-like structures have been observed in preparations of Tacaribe virus (Palmer et al., 1977), linear RNA molecules with some circular forms up to 1.5 μm in length were reported by Vezza et al. (1978a) for this virus. The possible mechanism whereby viral RNA is packaged within maturing arenavirus particles is more fully discussed in Chapter 6.

Although oligonucleotide analyses after T_1 nuclease digestion shows no homology between the large (L) and small (S) RNA segments, Auperin et al. (1982) have shown considerable homology at the 3′-termini of Pichinde isolate 3739 and the Munchique isolate of the same virus that has been adapted for virulence in guinea pigs (Chapter 5). This study showed that the L and S RNA 3′-terminal 19 nucleotides are essentially

identical with some minor exceptions. This nucleotide sequence includes 10 bases which represent an inverted identical sequence present on both L and S RNA molecules. These sites may be important for enzyme recognition prior to viral genome transcription and/or replication; nucleotide triplets which could represent mRNA initiation codons are present at positions 31–38 and 84–86 on the L and S strands respectively. As genetic reassortment studies have shown that the S strand contains gene sequences for Pichinde N, G1 and G2 viral proteins (Vezza et al., 1980; Leung et al., 1981), the gene order of these proteins may be determined by correlating terminal amino acid sequences of purified viral polypeptides with those sequences predicted by nucleotide sequencing.

Structures resembling 'pan-handles' have been observed in both Tamiami and Tacaribe RNA preparations, indicating RNA regions close to termini may form local regions of double-stranded RNA by complementary base pairing (Vezza et al., 1978a). Some confirmation of this for LCM virus has been suggested by Dutko et al. (1981) who found that full denaturation in the presence of glyoxal was temperature-dependent. Denaturation at temperatures below 60°C resulted in the appearance of additional L and S RNA bands in 1.5% agarose gels, the behaviour of which was compatible with the retention of some secondary structure. In addition, the circular nucleocapsids observed by Young and Howard (1983) may arise as a result of base pairing. A similar hypothesis for the formation of circular nucleocapsids has been suggested for the bunyaviruses (Obijeski and Murphy, 1977) supported by the demonstration of sequence complementarity between the 3′- and 5′-termini of the viral RNA segments (Parker and Hewlett, 1981). Given that circularization may occur as a result of end sequence base pairing at 3′-terminal ends of both L and S viral RNA (Auperin et al., 1982a); the variable lengths of nucleocapsid structures seen by Young and Howard (1983) suggests the possibility of multiple copies of either one or both RNA species within individual nucleocapsids. This concept is supported by the finding of Romanowski and Bishop (1983) that recombinants can be obtained by crossing ts mutants of LCM virus genetically diploid with respect to the S RNA segment. T_1 RNase digestion analyses of both RNA segments from the CA-1371 (Armstrong) and WE strains have been shown previously to allow each species to be readily identified from each other (Kirk et al., 1980). Among the recombinants obtained by crossing various mutants, one obtained by dual infection with an Armstrong mutant and a WE mutant both deficient in the S RNA generated wild-type virus that contained the L RNA of the Armstrong strain and S RNA oligonucleotides from both parent viruses. The progeny from this experiment was found to be unstable on passage at the permissive temperature of 35°C with sub-

sequent segregation of the ts viruses. Therefore it is to be concluded that this particular wild-type recombinant had arisen by gene-product complementation in virions diploid for the mutant S RNA species, evidence being presented in this study to exclude either a possible third genetic complementation group or wild-type plaques arising by aggregation of different mutant types. Although both S RNA species from the parent mutants could be readily distinguished, oligonucleotides of the S RNA from the Armstrong mutant predominated. Furthermore, segregation from the recombinant inevitably produced ts mutants with the S RNA of the Armstrong type, presumably as this genotype has some advantage in virus replication at the permissive temperature.

As discussed above, there are three primary gene products that have so far been associated with Pichinde virus infections: these are the major N protein component of the nucleocapsid, a high molecular weight 'L' component and the 'GPC' precursor of the two envelope glycoproteins. The N and GPC components (sum total molecular weight approximately 106 000) are gene products of the S RNA (Fig. 7.4a). The evidence for this is as follows. A recombinant virus containing the L RNA of Pichinde 3739 prototype strain and the S RNA of the Munchique variant (Vezza et al., 1980) was used to infect BHK-21 cells and virus-specific products differentiated by gel electrophoresis. The major N protein present co-migrated with the more rapidly migrating N protein of the parental Munchique variant, and the identity of the two proteins was confirmed by peptide mapping (Leung et al., 1981; Harnish et al., 1983). There appears to be differences of at least two major tryptic peptides (Vezza et al., 1980; Harnish et al., 1983). Similarly it was found that the GPC envelope precursor to polypeptide present in cells infected with the parental Munchique variant differed from GPC in prototype-infected cells. As the recombinant contained the S RNA species of the Munchique variant, it follows that the genes for both N and GPC proteins reside in the S RNA segment. It is also tentatively concluded from these studies that the L protein is coded for by the L RNA species, as its total molecular weight exceeds the coding capacity of the smaller S RNA segment. Harnish et al. (1983) have indeed found that tryptic peptide analysis can distinguish the L protein in cells infected with either wild-type Pichinde or Munchique viruses. The RE-2 recombinant used in these studies clearly possessed the L polypeptide similar to that present in the Pichinde parent virus. Leung and colleagues (1981) have predicted that one of the two groups of ts mutants obtained should contain lesions in the L protein resulting in defects in either transcription or replication or both if this protein is indeed responsible for these functions, although this has yet to be directly tested.

CHAPTER 8

Arenavirus replication

Arenaviruses replicate in a wide variety of mammalian cell types (Hotchin, 1971a), although biochemical studies are invariably restricted to the use of BHK-21 cells or various monkey kidney cell lines. Most arenaviruses will also grow well in mouse L cells but the simultaneous production of C-type retroviruses restricts their usefulness (Faras and Erikson, 1969). Maximum virus adsorption to cell surfaces occurs within 2 h at 37°C (Pfau, 1974), although it has been reported that an improvement may be obtained in the presence of DEAE dextran (Mifune et al., 1971). Growth kinetics are most thoroughly understood for Pichinde and LCM viruses. At multiplicities of infection below 0.1, the latent period is approximately 6–8 h after which time there is an exponential increase in cell-associated virus. Maximum titres of extracellular virus occur between 36 and 48 h after infection (Fig. 8.1; Mifune et al., 1971; Lehmann-Grube et al., 1975; Vezza et al., 1977). Significantly longer periods of time elapse before titres of other arenaviruses reach a maximum, although multiplicity of infection, temperature and the possibility of large amounts of non-infectious or defective interfering particles present in inocula all determine the growth kinetics of any particular arenavirus. Optimal yields of both Pichinde and LCM viruses are about 300 pfu per cell (Pedersen, 1979; Vezza et al., 1977). The number of infectious centres in any particular cell culture expressed as a percentage of the total number of cells varies widely; using immunofluorescence, Coto et al. (1974) estimated that only 3–6% of cells became infected with Junin virus as opposed to nearly 100% with LCM virus (Pedersen, 1970). How-

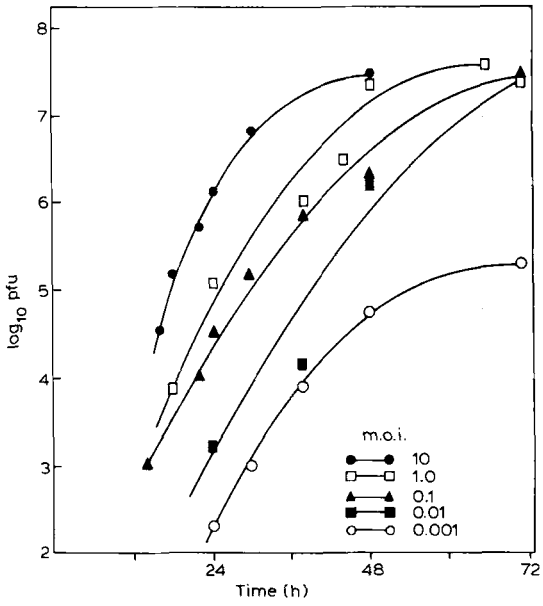

Fig. 8.1. Growth curves of Pichinde virus in BHK-21 cell cultures at different multiplicities of infection (moi). Virus yields are given as plaque-forming units (pfu).

ever, few direct measurements have been made in any one particular cell system; Pedersen (1979) has stressed that the passage history of any particular virus stock is probably one of the most critical factors determining the kinetics of arenavirus replication.

Infected cells undergo only limited cytopathic changes in commonly used cell lines with little or no change in the total level of host cell protein synthesis and virus yields may vary between susceptible cell types. There are few comparative studies using different cell substrates, one exception being the study of Lukashevich et al. (1983a). These authors found that Lassa virus grew to high titre in swine kidney cells in addition to Vero and L cells when similar high yields of virus were obtained in primary human embryo kidney cells. Lower yields were obtained in BHK-21 and CV-1 cells.

Persistently infected cell cultures may be readily established, individual cells being morphologically similar to uninfected cells and possessing

similar growth kinetics (Lehmann-Grube et al., 1969; Boxaca, 1970; Staneck et al., 1972; Welsh and Buchmeier, 1979).

8.1. Involvement of host cell components

Early studies clearly demonstrated that halogenated deoxyribonucleotide analogues failed to inhibit arenavirus replication (Pfau, 1974; Webb et al., 1967). In addition, no evidence was found for the presence of a virion-associated DNA polymerase (Carter et al., 1974). These results indicated that neither *de novo* DNA synthesis nor a DNA intermediate were required for arenavirus genome replication. However, the yield of infectious virus was markedly increased by infecting cells in the exponential phase of growth as opposed to using confluent cell monolayers (Pfau et al., 1973; Rawls et al., 1976). Conversely, the addition of actinomycin D at concentrations which inhibit cellular RNA synthesis reduced the yield of infectious virus (Buck and Pfau, 1969; Mifune et al., 1971; Stanwick and Kirk, 1971) suggesting that a cell function is required for optimum virus growth. Direct evidence for the requirement of an intact host cell nucleus for Pichinde virus replication has been provided by Banerjee et al. (1975/76) who found that BHK-21 cells previously enucleated by cytochalasin B treatment failed to support the production of infectious virus. Attempts to detect viral antigen synthesis by immunofluorescence proved negative. Enucleation of cells at varying times after infection indicated that the requirement of host cell nuclear products was restricted to the first 10 h after infection. Banerjee et al. suggested that virus-specific RNA synthesis occurs in close proximity to the nuclear membrane; cytochalasin treatment thus devoids the cell of a primary site of RNA and protein synthesis.

An alternative possibility is a requirement for a host nuclear product to regulate viral genome expression. Chanas (1982) has shown by immunofluorescence that Pichinde virus nucleocapsid protein may be detected within the nuclei of infected Vero cells as specific inclusions. The nuclear location of this reaction was confirmed by immunofluorescence using nuclear monolayers derived from infected cells treated with Nonidet P40. The antigen did not appear associated with the nucleolus and appeared transiently during infection, the time of appearance being dependent on multiplicity of infection. Using a monoclonal antibody against the larger envelope glycoprotein of Pichinde virus (G1) these studies also showed that the perinuclear area was of importance for glycoprotein synthesis; early after infection, this protein was detected initially in the perinuclear

area and was seen subsequently to spread through the Golgi complex situated close to the nuclear membrane.

The finding that the bicyclic octapeptide alpha-amanitin prevents the replication of both Pichinde and Tacaribe viruses (Leung, 1978; Mersich et al., 1979) suggests that cellular DNA-dependent RNA polymerase II plays a role in arenavirus replication. This nuclear enzyme is responsible for the synthesis of host cell heterogenous nuclear RNA and mRNA synthesis (Roeder, 1976) although it is worth remembering that at the concentration level required for inhibition of virus replication the activity of nuclear RNA polymerase III is inhibited (reviewed by Lewin, 1980). The latter enzyme appears responsible for synthesis of tRNA and other small RNA species (Weinman and Roeder, 1974) and has been shown to be responsible for transcribing small lengths of RNA from the adenovirus genome (Weinman et al., 1976). In contrast, RNA polymerase II activity is inhibited in mammalian cells at levels below 0.1 μg/ml, almost 100-fold less than the concentration required to inhibit Tacaribe virus replication in treated cells. The observations regarding the effect of actinomycin D on Pichinde virus replication show that, in contrast, virus replication is not inhibited under conditions where host rRNA synthesis is effectively prevented (Carter et al., 1973b). In fact, the addition of the drug in low concentrations results in an increase in amount of infectious virus although there is no apparent increase in the total number of virus particles. Under these conditions, RNA production is selectively blocked but synthesis of nucleoplasmic heterogenous RNA continues (Penman et al., 1968). Prolonged treatment of 48 h or more may, however, reduce virus yield (Martinez Segovia and Grazioli, 1969; Coto and Vombergar, 1969; Rawls et al., 1976). The decrease in virus yield of Pichinde virus at higher concentrations of actinomycin by Rawls et al. was not linearly related to the concentration of the drug but did parallel a decrease in RNA synthesis observed in control cells. Interestingly, there was no apparent decline in the percentage of cells expressing viral antigen and the extent of the inhibition decreased proportionally if actinomycin D was added at varying times after infection. Virus antigen expression at the surface of infected cells remained unaffected by the addition of actinomycin D, providing a further indication that higher concentrations of this compound may interfere with virus maturation. Similarly, Lukashevich et al. (1984) have successfully used actinomycin D at a concentration of 1 ng/ml which was found to prevent Machupo virus maturation but viral specific protein synthesis continued. Under these conditions, RNA of similar size to viral L and S strands was resolved against a reduced background of cellular RNA. The involvement of the host cell nucleus may

have consequences for the understanding of arenavirus persistence. Mims (1966) observed both cytoplasmic and nuclear immunofluorescence in LCM infected cells from chronically infected mice and Chanas (1982) demonstrated transient nuclear fluorescence in a low proportion of Vero cells persistently infected with Pichinde virus. However, the latter study also showed the appearance of viral antigens in the nuclei of acutely infected MDBK cells which do not become persistently infected with this virus, indicating that nuclear involvement *per se* may not necessarily always be associated with virus persistence.

The experiments with actinomycin D demonstrate that virus replication does not require newly synthesized ribosomal RNA; indeed ribosomal RNA synthesized prior to infection may subsequently be incorporated into new virions (Carter et al., 1973b). Leung and Rawls (1977) also showed that Pichinde virus-associated ribosomes were not required for virus replication. In these experiments, virus was first grown at 33°C in ts-14 cells, a line of hamster embryonic lung cells which do not synthesize proteins at 39°C owing to a ts defect in the 60 S ribosomal subunit. Virus grown under these conditions subsequently replicated successfully in wild-type hamster lung cell cultures at either 33° or 39°C. Ribosomes which become incorporated into nascent virus do, however, retain the capacity to catalyze protein synthesis (Chinault et al., 1981).

8.2. Synthesis of virus-specific polypeptides

The major nucleocapsid protein (N, average molecular weight 64 000) is stoichiometrically the most abundant polypeptide in extracellular virions and is the most predominant virus-specific product in the cytoplasm of infected cells as detected by immunofluorescence and metabolic radiolabelling. The failure of arenaviruses to shut off host cell protein synthesis has hindered the analysis of intracellular protein expression, in particular precursor-product relationships, and therefore there is greater dependence on the specific immunoprecipitation of virus polypeptides using polyclonal, or more recently monoclonal, antisera as compared to other viral systems. Buchmeier et al. (1978) demonstrated that the N polypeptide of LCM could be specifically immunoprecipitated from infected cells and was in the same molecular size range as the protein found within extracellular virus. The N protein was detected in the cell 6 h after infection, corresponding to the appearance of new infectious virus. Pulse-chase experiments showed no change in the electrophoretic properties of intracellular N polypeptide, indicating that cleavage of this protein did not

occur. The similarly sized N protein of Pichinde virus follows similar dynamics of synthesis (Dimock et al., 1982). Interestingly, the percentage of cells positive for N protein as detected by immunofluorescence increased concomitantly with virus production; however, most cells subsequently remained strongly positive even after virus production and virus-specific protein synthesis had declined by over 90%. This finding demonstrates the antigenic stability of this gene product, a finding that may be relevant in explaining the intensive fluorescence seen *in vivo* in tissues which show little or no evidence of virus particle production at the ultrastructural level.

LCM virus-infected tissues were shown as early as 1939 to contain an antigen with complement fixation (CF) properties (Smadel et al., 1939). Furthermore, this antigen was termed 'soluble' on the basis that the activity could be separated from whole virus by differential centrifugation. Antigenic activity was stable on storage but diminished on heating above 56°C (Smadel et al., 1940). Brown and Kirk (1969) described a similar heat-sensitive CF antigen in LCM-infected BHK-21 cells, a proportion of which was associated with the virus after centrifugation of cell extracts. Several studies have indicated that the major antigen detected by CF is identical to the antigen specificities detected in the cytoplasm by immunofluorescence (Buchmeier et al., 1977; Rutter and Gschwender, 1973). Bro-Jorgensen (1971) found two distinct precipitin lines between LCM antiserum and a sucrose-acetone extract of infected cells. The smaller of the two antigens was found to be thermostable, resistant to the action of trypsin, and of approximate molecular weight 48 000. Pichinde viral antigens in infected cell extracts can also be readily detected by CF tests 2–4 days after infection. Buchmeier et al. (1977) detected two virus-specific proteins by agar-gel diffusion. The predominant antigen was stable at 56°C and resistant to pronase; in contrast, the minor antigen was found to be thermolabile and destroyed by pronase treatment. Buchmeier et al. found that a purified preparation of the major, heat-stable antigen contained two polypeptides with molecular weights of 15 000 and 20 000, respectively. Serological identity was established between this preparation and a solubilized fraction of Pichinde virus containing the nucleocapsid; this disparity in size between the fraction isolated by Buchmeier et al. and the polypeptides within the infected cell cytoplasm may be attributable to the action of proteases in the cytoplasm of cells, particularly on the N polypeptide. Evidence to support this hypothesis was recently published by Harnish et al. (1981) who found up to six minor bands present in immunoprecipitates of Pichinde virus infected cells.

At 24 h following infection at a multiplicity of 1 pfu per cell, three of these minor components (molecular weights 48 000, 38 000 and 28 000, respectively) were related to the major 64 000 molecular weight N protein by peptide mapping, although the 28 000 molecular weight component did show a minor change in peptide structure. Additionally, three smaller bands in the molecular weight range of 14 000 to 17 500 were also found to be derived from the N protein and may be analogous to bands characterized earlier by Buchmeier et al. (1977) as having CF ability. Although these additional minor bands reported by Harnish et al. (1981) may represent proteolytic cleavage products arising during extraction, the authors point out that such products are evident with a radiolabel chase period of as little as 10 min, suggesting either that proteolysis occurs at a very rapid rate or that they represent discrete premature termination products of N protein mRNA translation. Alternatively, they may represent full translation products of mRNA species transcribed from within the N gene. The appearance of N-related products in Pichinde-infected cells represents a major difference compared to LCM infection, discussed earlier where there is no evidence of such cleavage products. However, a recent study suggests that cleavage of N may occur in cells infected with Lassa virus, another Old World arenavirus (Clegg and Lloyd, 1983). It remains to be clarified as to whether these findings represent intracytoplasmic cleavage events or occur during extraction and subsequent analysis. Dimock et al. (1982) suggested that continuing N antigen expression in infected cells after the decline in virus production may represent the continuing presence of a low molecular weight cleavage product of the N protein which is protease resistant.

As cells retaining arenavirus antigens appear to be resistant to reinfection with homologous virus (Hotchin, 1973) they speculated that such a product of N protein cleavage may inhibit the expression of the viral genome.

There is some suggestion that a slightly larger protein bearing similar chemical sequences to N may be present in Tacaribe infected cells. The nucleoprotein band is synthesized as a major gene product from 24 h after infection in infected BHK-21 cells, reaching a maximum at 48–60 h (Saleh et al., 1979). In addition, a minor 79 000 mol wt (previously identified by the authors as 'P') was also detected in immunoprecipitates prepared from infected cell extracts during the same period and gave similar digestion products compared to N protein on limited digestion with *Staphylococcus aureus* V8 protease.

There is some evidence that the N polypeptide is phosphorylated in the extracellular state (Gimenez et al., 1983). In addition, Howard and

Buchmeier (1983) have recently demonstrated that LCM virus particles contain an endogenous protein-kinase activity which is capable of phosphorylating the N polypeptide. After phosphorylation, this viral component migrates slightly faster in acrylamide gels. The enzyme remains bound to separated nucleocapsids, and it is tempting to speculate that some alteration of nucleocapsid structure which would allow primary transcription of the arenavirus genome may be effected by phosphorylation early in infection. Alternatively, this activity may have some regulatory effect on the viral RNA polymerase in a manner similar to that proposed for the protein kinase associated with vesicular stomatitis virus (Witt and Summers, 1980). This does not exclude the further possibilities that phosphorylation, and subsequent dephosphorylation, may play a role in determining the fate of nascent N polypeptide molecules: those with a strong net negative charge would be unlikely to be used for nucleocapsid assembly until removal of phosphate moieties, if present. Phosphorylation of this and maybe other virus-specific polypeptides may occasion their dual role as regulatory, as well as structural proteins.

As discussed in the previous chapter, it has been demonstrated that both N and the envelope proteins are products of the S RNA strand (Leung et al., 1981; Riviere et al., 1985). However, as the number of copies of N present in extracellular virus outnumbers the sum of the two glycoprotein species by a ratio of about 3 to 1, there must be a control effector mechanism either at the level of translation of virus-specific mRNA or during virus maturation. Given the finding that viral antigen within infected cells is predominantly N associated, it seems likely that there is a real difference in rate of synthesis, although preferential cleavage of glycoproteins not required for maturation or their transport from the cell into extracellular fluid as free molecular entities cannot be excluded at the present time. Analysis of LCM infected cell extracts showed that cells infected with this virus contained a 75 000 molecular weight glycoprotein which was not present in extracellular virus particles (Buchmeier et al., 1978; Buchmeier and Oldstone, 1979). Two-dimensional peptide mapping and pulse-chase experiments revealed that this entity, identified as GPC, represented a precursor molecule of the two envelope glycoproteins present in mature LCM virions. Optimal detection of this precursor by metabolic radiolabelling was found to be dependent on both the time of addition of radioisotopes and multiplicity of infection. The glycosylation profile of the two cleavage products closely resembled that seen in the G1 and G2 glycoproteins recovered from extracellular virus, being comparatively rich in glucosamine and fucose. The smaller G2 molecule additionally contained galactose. In contrast,

the cellular precursor was rich in mannose and glucosamine but contained only minor quantities of fucose and galactose. These findings suggest that maturation of the LCM envelope glycoproteins is accompanied by modification of sugar residues, probably by trimming of mannose-rich single-chain carbohydrate structures and subsequent addition of multiple-branched complex sugar chains as has been described for Tacaribe (Boersma et al., 1982) and other enveloped viruses, for example, vesicular stomatitis virus (Etchison et al., 1977). There is no evidence that the arenavirus glycoproteins are sulphated (Vezza et al., 1977; Harnish et al., 1981).

A similar precursor-product relationship between a high molecular weight glycoprotein found only in the cytoplasm and the two envelope glycoproteins of Pichinde virus has been described (Harnish et al., 1981). In the presence of tunicamycin, the molecular weight of 'GPC' present in infected cells was reduced from 79 000 to 42 000, indicating that as much as half of the precursor molecule may consist of unbranched sugar chains. The sum total molecular weight of primary gene products expressed from the Pichinde S RNA segment is therefore approximately 100–110 000, in accord with the estimated size and coding capacity of this genome fragment (see Chapter 7).

Viral antigens, particularly the 44 000 molecular weight G2 glycoprotein first appears on the surface of LCM infected cells from 7.5 h after infection and increases in intensity until greater than 90% of cells are intensely stained by fluorescent antibody 48 h after infection (Buchmeier et al., 1978), reflecting an accumulation of virus-specific material at the cell surface. In contrast, viral nucleoprotein is never expressed at the surface of arenavirus-infected cells. These studies were complemented by the finding at the plasma membrane of the larger 54 000 molecular weight G1 protein after lactoperoxidase-catalyzed surface iodination of infected cells (Buchmeier and Oldstone, 1979). It is possible that the different results obtained by the two approaches may reflect different spatial arrangements of the envelope glycoproteins restricting the number of tyrosine residues available for iodination (Buchmeier et al., 1978) as both glycoproteins of Pichinde virus may be identified by similar radiolabelling of extracellular particles (Young, 1985). The dynamics of Pichinde glycoprotein synthesis appear to follow closely the appearance of nucleoprotein during the early stages of infection, in accord with the concept that both of these gene products arise by transcription of the same viral RNA segment (Dimock et al., 1982). Occasionally, high molecular weight bands may be detected in analytical acrylamide gels of viral proteins. Among these structures a band ('L') of approximately 200 000

molecular weight has been consistently seen in purified preparations of Pichinde virus (Harnish et al., 1981; Young, 1985). It has been suggested that this may represent a product of the L viral RNA segment as its molecular weight exceeds the coding capacity of the S RNA segment and does not share any common peptide sequences with other Pichinde virus gene products. Further evidence as to the virus-specific nature of this band is its appearance within infected cells at the same time as N and the glycoprotein precursor (Dimock et al., 1982). However, there is no indication yet as to the precise nature and function of this protein, although it is tempting to speculate that this may possibly represent the virion-associated RNA polymerase activity. Certainly, high molecular weight bands may be found associated with purified nucleocapsids, although in the absence of purified enzyme or specific antibody probes it is not possible to ascribe particular functions to these additional structural components present in minor amounts. However, the virus specificity of the L protein is suggested by its absence in uninfected cells, its appearance simultaneously with other viral gene products, and quantitatively its synthesis appears related to the multiplicity of infection (Harnish et al., 1981).

8.3. RNA synthesis and genome replication

Only limited information is available concerning the replication and expression of viral RNA within infected cells, although direct nucleotide sequencing of the complete genome is now possible and new information as to possible replication events can be predicted from an examination of these sequences. The availability of cDNA probes to both L and S Pichinde RNA segments (Leung et al., 1981) allowed Dimock et al. (1982) to follow the appearance of virus-specific polypeptides. This study suggested that the peak of virus-specific polypeptide synthesis coincided with the peak expression of both L and S strands in the cytoplasm of infected cells 2 days after infection at a multiplicity of 0.1 pfu per cell. Several problems inherent in this type of approach were highlighted by this study: the first is that hybridization measures only the availability of RNA template available for virus-specific mRNA. No information is available regarding RNA strand stability or relative rates of transcription from the viral genome. The second is the recurrent problem that materials separated on agarose or acrylamide gels are frequently contaminated with small amounts of ribosomal RNA or indeed each viral RNA band may be contaminated by trace amounts of the heterologous species. However, it is likely that control mechanisms do operate as the

kinetics of viral gene expression and replication appears to be independent of the cell cycle indicating that the decline in virus production seen by Dimock et al. (1982) after 48 h represents a control mechanism mediated either by viral gene products or, less likely, a cellular factor.

A major point that remains unresolved concerns the properties of the endogenous RNA polymerase that is presumably required for initiation of primary transcription during the early stages of infection. Are host or virus-induced modifications required prior to transcription? Is that same enzyme responsible for both plus and minus strand synthesis? As with the study of arenaviral RNA species, data concerning the nature of the endogenous RNA polymerase present in extracellular virions is hampered by the simultaneous presence of host ribosomes with associated polymerase activities. In an early report, Carter et al. (1974) reported that total RNA polymerase activity in Pichinde virions was optimal in 10 mM Mg^{2+} at pH 8.5 and that the addition of actinomycin D *in vitro* was without effect whereas the inclusion of pancreatic RNase abolished enzyme activity. The product of this reaction consisted of two components; a low molecular weight 6 S component which was RNase susceptible and a much larger 26 S RNA component which was partially RNase resistant. The nature of the Pichinde virus-associated RNA polymerase activity was carefully analyzed further by Leung et al. (1979), who found that whilst RNA polymerase associated with viral ribonucleoprotein structures catalyzed the incorporation of all four ribonucleotides, enzyme activity present in the slowly sedimenting fraction which contained predominantly ribosomal material preferentially catalyzed the incorporation of AMP and UMP nucleotides. This latter activity represented two discrete enzymes, one preferentially catalyzing UMP in the presence of Mg^{2+++}, and a second Mn^{2+} dependent activity which preferentially incorporated AMP. Both activities closely resemble similar RNA polymerase moieties associated with uninfected cell ribosomes. A further finding was the probable presence of a nucleoprotein-associated RNase, as manifested by a decrease of acid-precipitable radiolabel after 10 min of RNA polymerase activation. The RNA polymerase associated with the virus-specific ribonucleoprotein appeared to be much more labile than the two host-derived enzymes, and RNA-RNA hybridization showed that only about 23% of the virion RNA was copied by the *in vitro* reaction. When this product was added to the fraction containing the Mn-dependent polyadenylate polymerase activity, a homopolymer of poly A was terminally added to the virus plus-stranded RNA product of the first reaction. It is unclear if this has any functional significance as polyadenylation of viral mRNA at the 3′-terminal early during infection presumably could be

effected by enzymes present in the newly infected host cell. It is also worth noting that the mRNA coding for at least some viral proteins is not polyadenylated (Auperin et al., 1984b). Although the protein synthesizing function of incorporated ribosomes is not required for initiating virus replication (Leung and Rawls, 1977), the ts host ribosomes used to demonstrate this property still contained fully active polymerase activity at the non-permissive temperature (Leung et al., 1979). Therefore a function of incorporated ribosomes requiring a non-protein synthesizing activity remains to be excluded.

The molecular cloning of the Pichinde and LCM virus S RNA genome segments has revealed further information as to the possible strategy of arenavirus replication. Sequence analysis from the 3′-end of the negative sense viral RNA molecule has shown an open reading starting at position 84 which represents the gene for the nucleocapsid protein N (Auperin et al., 1984a). A mRNA species coding for this polypeptide was found in extracts of infected cells and the location of this transcript with respect to the viral genome confirmed by hybridization with cDNA representing the sequenced region. Therefore, assuming primary transcription occurs at the 3′-end of the parental genome, production of N polypeptide appears to be the first structural protein synthesized from the S strand during virus replication. This result is in good agreement with the work of Leung et al. (1984) who synthesized a short peptide representing the first eight amino acids of the predicted sequence at the NH_2-terminus of the polypeptide and found antibodies to this synthetic peptide specifically reacted with the N protein found in infected cells. An examination of the sequence towards the 5′-end of the gene shows the presence of a termination codon at position 1767. The predicted length of this mRNA is compatible with the size of the N polypeptide obtained by gel electrophoresis and suggests that N represents a primary gene product. However, it is known that at least one minor protein band with slightly higher molecular weight and containing at least some N peptide sequences is frequently present within extracellular virions (Compans et al., 1981). The possibility of limited read through past this termination codon remains a possibility and therefore a proportion of N may arise by subsequent post-translational cleavage. The messenger for N protein may not be polyadenylated as there are no U-rich sequences flanking the end of the gene which could serve as a polyadenylation signal and mRNA extracted from infected cells apparently does not bind to oligo-dT cellulose (Auperin et al., 1984b). This point remains to be clarified, however, as there is some evidence of poly A tracts being present at the 3′-ends of viral mRNA in cells infected with LCM virus (P. Southern, personal communication).

Further examination of the Pichinde viral S RNA sequence shows a gap of some 60 nucleotides before a further putative translation initiation codon is encountered. Although it is conceivable that the subsequent region may code for a viral protein, examination of a complete set of

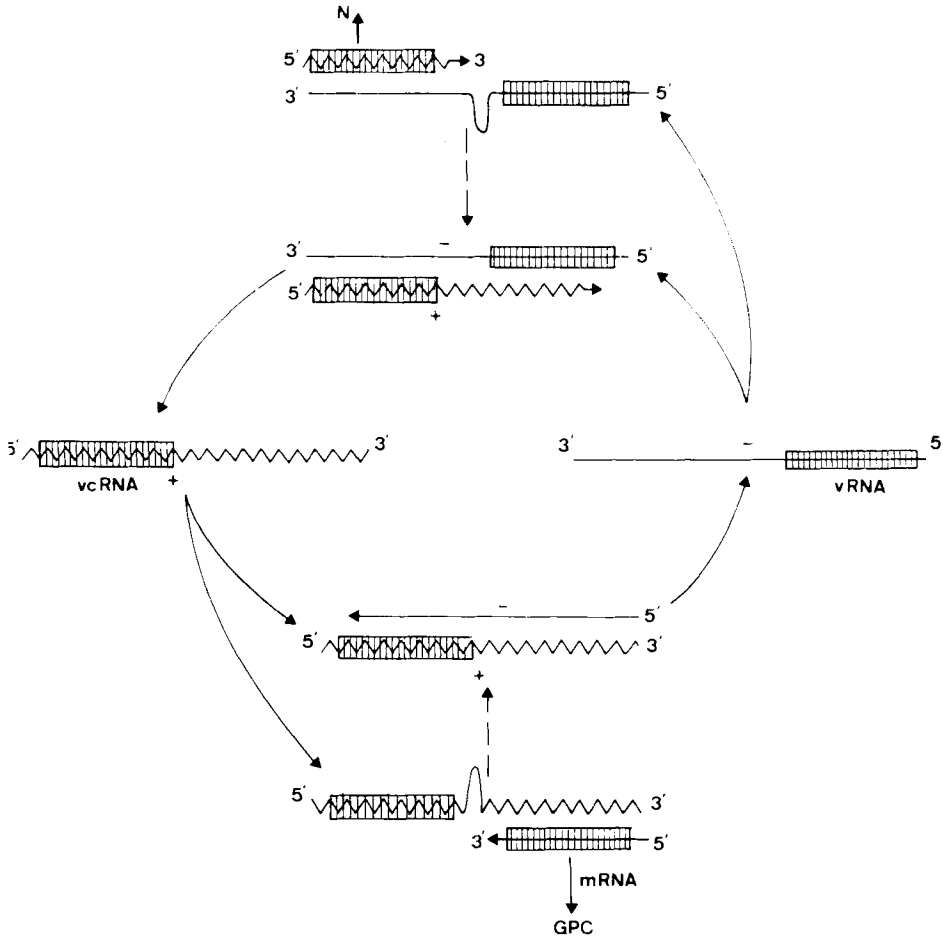

Fig. 8.2. Strategy of arenavirus genome replication and expression as proposed by Auperin et al. (1984b). Although the viral RNA is not infectious *per se*, this model implied the S strand contains a messenger-sense GPC gene at the 5′-end of the genome which requires a full replication cycle for expression. In contrast, the negative sense N gene (open box) may be expressed independently by limited RNA polymerase activity on viral RNA templates and does not require complete complementary strand RNA synthesis. Thus the 'ambisense' of the S RNA strand may allow the independent expression of these two major viral proteins.

cDNA clones encompassing the entire S RNA molecule has shown that expression of the arenavirus genome may follow a novel mechanism (Auperin et al., 1984b; Romanowski and Bishop, 1984). Examination of the complete sequence representing the complementary strand of positive polarity with respect to the N gene showed the absence of an open reading frame of sufficient length to code for the glycoprotein precursor GPC which genetic studies have conclusively shown is a product of S RNA gene sequences (Leung et al., 1981; Vezza et al., 1980; Harnish et al., 1983). However, the sequence representing the viral RNA of negative polarity does show an open reading frame starting at position 52 from the 5′-end, leading to the concept that the GPC molecule is coded in a subgenomic, viral sense mRNA species representing the 5′-end of the viral S RNA. The term 'ambisense' RNA has been coined to describe this form of genetic expression (Auperin et al., 1984b), and is schematically illustrated in Fig. 8.2. A similar mechanism of transcription for the envelope glycoprotein has also been proposed for the segmented genome RNA of the bunyavirus Punta Toro (Ihara et al., 1984). This suggests that arenaviruses possess independent mechanisms for expression of N and the envelope glycoproteins, thus explaining why the appearance and respective molar quantities of these gene products are not necessarily related. Independent expression of N protein, known to occur both late in acute infection and in persistently infected cells in the absence of low levels of glycoprotein production (see Section 8.4) would thus be explained by the production of subgenomic mRNA from negative polarity, virus sense template. A control mechanism must therefore exist which determines the fate of nascent, negative polarity RNA as being either destined for encapsidation or as a template for N protein-specific mRNA. In contrast, the template for glycoprotein-specific mRNA is of complementary sense to viral RNA and as such would not be required for nascent virus production. The lack of glycoprotein late in the replicative cycle (Dimock et al., 1982) or in persistently infected cells (Welsh and Oldstone, 1977; Oldstone and Buchmeier, 1982) would therefore imply selective transcriptional or translational control of this gene product. Control may also be mediated at the level of association between nucleic acid and N protein, although the high RNase sensitivity of viral RNA as nucleoprotein may still allow specific enzyme recognition.

Both viral RNA and its complementary strand contain hairpin sequences immediately following the GPC and N coding frames respectively (Auperin et al., 1984a). These may allow some control over the expression of both genes, for example by providing recognition points for termination of transcription by viral RNA polymerase (see Fig. 8.2).

Both hairpins are of almost identical composition on both strands, consisting of 18 base pairs; however, this sequence is not conserved between arenaviruses, LCM virus having a distinct 21 base pair intergenic hairpin (Romanowski and Bishop, 1985). At the time of writing, these sequences were still being evaluated and questions as to the possible mechanism of control will also require the development of a suitable *in vitro* system for the study of viral RNA polymerase activity. The nucleotide sequence in the hairpin region is of coding sense and may be translated, either via a discrete mRNA species or as a result of extended transcription of N or GPC messengers through this region. The small, so-called 'VP4' structural protein (Ramos et al., 1972) seen subsequently by many investigators has yet to be assigned to a reading frame on the viral genome and may represent transcription of the intergenic region on the S RNA molecule. The binding of ribosomes to the 5′-ends of both viral and complementary arenavirus RNA is predicted by the sequence of nucleotides flanking the initiation region; these contain an A or G base three nucleotides upstream and a G base immediately after the AUG triplet (Kozak, 1983). So far the postulated reading frames for viral gene products transcribed from LCM and Pichinde genomes fit these rules (Romanowski and Bishop, 1984). In addition, the preferred sequence for ribosomal 18S subunit binding is present on both putative mRNA molecules (Young, 1985).

It is widely assumed that the viral RNA polymerase is encoded by the L RNA strand, although formal demonstration of this is still lacking. The availability of nucleotide sequence for this larger genome segment (for example, Romanowski and Bishop, 1985) allows the prediction of amino acid sequences of particular reading frames and subsequent synthesis of short peptides for production of animal antisera, so the reader may expect rapid development in this area within the next few years. The similar 3′- and 5′-nucleotide sequences at the ends of both L and S RNA species imply common recognition mechanisms by the viral RNA polymerase holoenzyme although early in replication there is presumably some preference for transcription of the L strand in order that the pool of viral RNA within the infected cell may be expanded as a result of primary transcription. A requirement for an intact host cell nucleus during the early stages of replication (see Section 8.1) suggests either a host RNA or protein primer may be required, at least for these early RNA events. In common with the S RNA strand, initiation codons identified on the L strand possess the flanking nucleotide sequences favoured as eukaryote translation initiation signals.

8.4. Replication in persistently infected cells

Persistently infected cell cultures may be readily established using most of the cell lines suitable for virus growth, with the possible exception of MDCK cells (Dutko and Pfau, 1978; Chanas, 1982). Indeed, some cell substrates commonly used for the study of other virus systems may become unintentionally contaminated with arenavirus and the infection persist with little or no cytopathology. One such instance has been described recently by van der Zeijst et al. (1983a, b) who found that a previously unidentified antigen present in a line of SV40 transformed BHK-21 cells was in fact the nucleocapsid antigen of LCM virus. The appearance of this antigen was associated with intracellular infectious material that could be transmitted by cell-free extracts to susceptible animals including hamsters although extracellular virus *per se* could not be demonstrated. This example serves to illustrate that persistently infected cell cultures may be more ubiquitous than previously supposed, with a particular risk of LCM virus contamination in virus systems where material is frequently passaged through mice or hamsters. Persistence is often associated with the production of large quantities of interfering virus particles and homotypic viral interference may readily be demonstrated with LCM (Lehmann-Grube, 1971; Hotchin, 1971a; Welsh and Pfau, 1972, Pfau, 1977) Amapari and Parana (Staneck et al., 1972; Welsh and Pfau, 1972), Junin (Coto, 1974; Damonte et al., 1983), Pichinde (Dutko et al., 1976) and Tacaribe viruses (Gimenez and Compans, 1980).

The phenomenon of interference does not appear to be strain-specific as was shown by Welsh and Pfau (1972) who showed interference between all possible pairs of LCM virus strains CA-1371 (Armstrong), M-7, UBC and WE. This homologous interference was demonstrated both at the cytolytic level in infected cells, the number of infective centres and the level of virus production. These and subsequent studies to be discussed below showed there is no evidence of interference mediated by interferon; the early studies of Welsh and Pfau showed that the interfering material is heat-labile with all the physical and chemical properties of virus particles. In addition, the interference effect is restricted to members of the arenavirus family, there being no difference in the capacity of persistently infected and control cells to support the replication of other viruses, e.g. Sindbis, VSV and picornaviruses.

In certain circumstances, heterotypic interference between arenaviruses can be demonstrated (Welsh and Pfau, 1972; Staneck and Pfau, 1974, Damonte et al., 1983) and the degree of interference often parallels

TABLE 8.1.
Ability of persistently infected Vero cells to support replication by heterologous arenaviruses (after Damonte et al., 1983)

	Superinfecting Virus					
Cells	Junin	Tacaribe	Amapari	Pichinde	Tamiami	LCM
Control	++++	++++	++++	++++	++++	++++
Junin	-	-	-	++++	++++	++++
Tacaribe	-	-	-	++++	++++	++++
Pichinde	-	-	-	-	++	++
Tamiami	-	-	-	-	-	-

++++ = 4 Logs or more of virus yield.
++ = 2 Logs or more of virus yield.
- = No virus growth.

comparative serological studies between different arenaviruses (see Chapter 3). The study of Welsh and Pfau (1972) clearly showed that high concentrations of LCM virus resulted in fewer and less well-defined plaques due to either Amapari or Parana viruses when plated simultaneously on L cells. These experiments were expanded by Staneck et al. (1972) who found that BHK-21 cells positively infected with LCM virus were also resistant to Tacaribe in addition to Amapari virus. Parana virus was also used to establish persistently infected cells and in this instance there was complete resistance to Tamiami and LCM virus.

The variable response of persistently infected cultures to infection with heterotypic arenaviruses has been carefully documented by Damonte et al. (1983). Lines of Vero cells persistently infected with Junin, Tacaribe, Tamiami and Pichinde viruses respectively were superinfected with different arenaviruses after 13 passages at 3-day intervals. Cultures persistently infected with Junin virus were totally resistant to superinfection with Tacaribe and Amapari viruses, an observation that closely parallels cross-protection studies in laboratory animals susceptible to lethal Junin virus infection (Weissenbacher et al., 1975/76, 1982). In contrast, these cells were able to support the replication of Pichinde, Tamiami and LCM viruses albeit with a lower yield of virus as compared to uninfected Vero cells. These findings are shown schematically in Table 8.1. The results obtained with cultures persistently infected with either Pichinde or Tamiami viruses were more complex, partly due to the spontaneous production of infectious virus from these cells. For example, although Tamiami

and LCM viruses were able to plaque directly in the Pichinde persistently infected cell cultures, the efficiency of plaque formation was markedly inhibited. It was also observed that in both systems, superinfection with heterotypic virus resulted in a drop in the level of persisting virus. The resistance to superinfection observed with cells persistently infected with either Tacaribe or Junin viruses appeared to be due to a form of interfering particle, although more than one mechanism may operate as it was noticed that Junin interfering particles failed to inhibit fully the replication of standard Amapari virus even though the latter virus was unable to replicate in cells persistently infected with Junin virus. Irradiation of Junin interfering particle preparation with ultraviolet light did not significantly reduce the interference seen against standard Junin or Tacaribe virus preparations, suggesting that if such interference is mediated at the genetic level, the interfering particle contains a reduced complement of radiation-sensitive nucleic acid may represent defective interfering (DI) particles.

DI particles are defined as a class of virus mutants dependent on standard virus for propagation as a result of a deletion of part of the viral genome (reviewed by Huang and Baltimore, 1977). In all systems so far, DI particles possess all the structural proteins found in standard virus. This has been proven for LCM virus, where no difference could be found in the polypeptide profile of DI particles released from persistently infected Vero cells compared to standard virus (Welsh and Buchmeier, 1978), and Tacaribe virus (Gimenez and Compans, 1980). However, Gimenez and Compans noticed that the major N polypeptide present in DI virus produced in BHK-21 cells persistently infected with Tacaribe migrated at a lower electrophoretic rate in acrylamide gels compared to standard virus. It was not clear whether this represents a modification of the major nucleocapsid protein or that N polypeptide present in standard virus is the result of a minor proteolytic cleavage event which does not occur in persistently infected cells. In any event, this difference was not seen in DI particles produced by persistently infected Vero cells. There appears to be no alteration in the antigenic nature of the DI particle glycoproteins. Several studies have clearly shown that both hyperimmune animal and human convalescent antisera to LCM virus reduce viral interference, confirming that DI particles share common antigenic determinants (Welsh and Pfau, 1972; Dutko et al., 1976; Popescu et al., 1976).

Somewhat different results have been reported for LCM virus particles produced by persistently infected L cells (Martinez Peralta et al., 1981).

Working with the WE strain of LCM, these authors have suggested that interference is mediated by an 'interfering particle' that replicates in parallel with standard virus, and is not defective in the conventional sense in that it requires the help of standard virus for passage and propagation (Lehmann-Grube et al., 1981). Although such interfering particles contained the major nucleocapsid protein, the two envelope glycoproteins were barely detected but a new glycoprotein species of molecular weight 65 000 was clearly seen and was immunoprecipitated by specific antibody.

Van der Zeijst and colleagues (1983a, b) have studied the synthesis of proteins and viral structures in BHK-21 cells persistently infected with LCM virus and have found both the major nucleocapsid protein (N) and the GPC precursor of the envelope glycoproteins, G1 and G2. Interestingly, the GPC precursor polypeptide could not be found on the surface of persistently infected cells, in contrast to previously published findings in acutely infected BHK-21 cells (Buchmeier and Oldstone, 1979). The larger of the two envelope glycoproteins (G1) was resolved, despite evidence that extracellular virions were not produced by the persistently infected cultures under study. The significance of this observation is not clear, except the authors suggest that a possible defect in the ability of GPC to be cleaved during virus assembly could be a factor in explaining the absence of virus maturation at the plasma membrane of these cells. The findings by Padula and Martinez Segovia (1984) and Bruns and Lehmann Grube (1984b) that neither Junin nor LCM virus grown in the presence of tunicamycin are non-infectious would also support the concept of GPC cleavage being essential for virus maturation. Other analyses of viral proteins revealed a number of larger proteins in persistently infected cell extracts with molecular weights in the range of 89 000 to 112 000; these may correspond to hitherto unidentified non-structural proteins, the level of which are enhanced during persistent infection, or precursors of structural proteins the processing of which is blocked or slowed as compared to acute infection. Certainly, it is possible that at least some of these higher molecular weight components may correspond to the additional protein bands described by Bruns et al. (1983a) in extracellular LCM virions.

One difficulty in assessing the relative particle types produced in cells persistently infected with arenaviruses is the difficulty in separating infectious from antigenically similar, but non-infectious particle types. LCM DI virions have a slightly lighter buoyant density value of 1.13 g/cm^3 compared to 1.14 g/cm^3 in linear density gradients of Renografin (Welsh and Buchmeier, 1979), but are insufficient for complete separation

per se. Similarly, both Pichinde and Tacaribe DI virus particles band at slightly lower density in sucrose (Dutko et al., 1976; Gimenez and Compans, 1980), but the difference is insufficient to effect a complete separation. Martinez Peralta et al. (1981) suggested the use of a very shallow Urografin gradient although this would only be successful if preceded by rigorous purification as material of interest was removed either from the bottom or top fractions of the gradient.

Although possessing the complete profile of structural proteins, such small differences in buoyant density suggest that DI particles differ slightly in total chemical composition, and indeed this is a feature of DI particles in other virus systems (Von Magnus, 1954). Current thinking is that DI particles contain deletions in the S viral RNA segment and/or smaller viral RNA molecules. However, there is a lack of agreement between analyses carried out in different laboratories and between arenaviruses. Dutko et al. (1976) found that S RNA was absent in Pichinde DI particles, but after 175 passages of persistently infected cultures DI particles containing a smaller, 20 S RNA species became apparent. This is in contrast to the RNA profile of Tacaribe DI virus particles which were found to contain neither the L nor the S RNA of standard virus but instead five smaller RNA species in the molecular weight range 0.2 to 1 x 10^6 (Gimenez and Compans, 1980). It is not clear in the latter study as to the origin of each of the five smaller bands or indeed the coding polarity of these new RNA species. Dimock et al. (1982) has found two new species appearing late in acute Pichinde virus infection with molecular weights of 0.5 and 1.0 x 10^6; however, neither of these species specifically hybridized with cDNA probes prepared using L and S viral RNA, even though the smaller RNA bands were found in extracellular virus from as early as 5 days after infection. These results were clarified by obtaining recombinant cDNA clones, however, when it became apparent that the smaller RNA species were derived from the 3'-end of the S RNA (Leung et al., 1984). Strand-specific hybridization confirmed the subgenomic nature of this RNA species 1.9 kb in length and the authors have suggested that this may be amplified in persistently infected cells at the expense of full length S RNA synthesis. This would offer an explanation for the lack of viral glycoprotein expression in such cultures. The nature of the larger RNA species 3.1 kb long was not confirmed, although the simultaneous appearance of both small RNA molecules would suggest a possible function in continuing to provide gene information for viral RNA polymerase synthesis.

Using an immunoprecipitation procedure for isolating viral ribonucleoprotein complexes for infected cells, van der Zeijst et al. (1983b) have

described two new RNA species in cultures persistently infected with LCM virus. The larger of these, estimated at 3.9 kb in length and intermediate in size between virion L and S RNA, may represent a DI-RNA derived from the L RNA molecule, but no direct proof of this was obtained. Similarly, the authors suggest the second, 2.7 kb RNA may be derived from the S RNA of standard infectious virus. Neither species possessed the characteristics of mRNA. It is difficult at present to compare these findings with the observations of other workers, save to conclude that arenavirus persistence is associated with the appearance of new RNA species which may represent deletions of certain specific gene sequences found in fully infectious virus particles. However, it should be remembered that fully infectious virus may be recovered from persistently infected cultures passaged many times (Staneck et al., 1972), indicating that in certain circumstances the complete genetic complement of the virus may persist, but perhaps in low copy number below the threshold of detection.

The interfering property of arenavirus DI particles is known to be more resistant to the effect of non-ionizing radiation, e.g. ultraviolet light as compared to standard virus, which also indicates that interference may be associated with the expression of RNA with a correspondingly reduced target size (Welsh et al., 1972; Staneck and Pfau, 1974; Damonte et al., 1983). Pedersen (1979) described an analysis of DI particles obtained from cells persistently infected with LCM which lacked completely the S RNA genome segment but contained approximately 94% of the L strand. Pedersen has also pointed out that on review of other findings concerning DI particle RNA profiles there is evidence of a minor reduction in the size of the L RNA genome segment found in arenavirus DI particles. The RNA analysis of Pedersen was performed on LCM DI particles with a higher, not lower, buoyant density value in sucrose as compared to standard virus (1.23 g/cm^3 versus 1.18 g/cm^3, respectively). The size of this DI particle was correspondingly less and may be similar to particles of reduced size previously seen within LCM infected cells by electron microscopy (Mannweiler and Lehmann-Grube, 1973) and the smaller 55 nm DI particles from Tacaribe persistently infected cells (Gimenez and Compans, 1980). The increased density in sucrose gradients compared to the more generally observed lighter density observed for DI particles in Urografin are explained by Pedersen as resulting from the varying effects of hydration of RNA in the two media.

Persistently infected cell cultures are usually obtained by an initial infection at a high multiplicity of infection (10 pfu per cell or greater). Cytopathic effects may or may not be seen according to the choice of cell

type, but in all cases chronic infection is accompanied by a lack of gross morphological change compared to uninfected cells. As with other standard virus-DI virus systems, a cyclical pattern of standard and interfering particle production can be detected in early passages. Ultimately, cells become permanently established with cellular growth characteristics resembling those of normal cells (Staneck et al., 1972; Popescu et al., 1976; Stanwick and Kirk, 1976). Although viral antigen may be detected in the cytoplasm, expression of viral antigen at cell surfaces is low as detected by immunofluorescence (Cole and Rutter, 1973, Welsh and Oldstone, 1977). Although DI particle production is high, there is an almost negligible lack of standard infectious virus suggesting that DI particles may be assembled and released from internal cell membrane surfaces. Interference may be typically quantitated by determining the percentage of infectious centres produced by standard virus in the presence of DI particles compared to control cultures receiving standard virus only (Welsh et al., 1972; Welsh and Buchmeier, 1979). The degree of inhibition is linearly related to DI particle concentration, interference in infectious centres having been shown to follow single hit kinetics with a sensitivity limit of 10^6 particles per ml (Welsh et al., 1972; Dutko and Pfau, 1978; Popescu and Lehmann-Grube, 1976). This procedure allows the expression of DI virus concentration in terms of the number of interfering units that each cell is exposed to in the presence of standard, infectious virus. Such a method generally requires prior concentration of test samples prior to assay, however. Gimenez and Compans (1980) used the simpler approach of estimating the extent of plaque-number reduction in the presence of interfering activity, but this allows only a qualitative estimation of DI particle presence owing to the much lower titre of challenge standard virus added to each culture.

Treatment of cells with DI virus preparations up to 4 h prior to challenge with infectious LCM virus was found to inhibit the production of the viral nucleocapsid (Welsh and Buchmeier, 1979). Conversely, addition of DI particles to infected cells 4 h or later following infection with standard virus failed to inhibit expression of the standard viral genome; additionally DI virus infection alone failed to induce the production of the major N nucleocapsid protein. Taken together, these results suggest that interference occurs during the period of early viral protein synthesis and that DI particles do not have an independent capacity to produce one of the major gene products normally coded for by the standard viral S RNA species. There is no evidence to suggest that interferon plays a role in the initiation and maintenance of cell cultures persistently infected with arenaviruses (Welsh and Pfau, 1972; Gimenez and Compans, 1980).

Other mechanisms have been proposed as additional to, or a prerequisite for, the establishment and maintenance of arenaviral persistence *in vitro*. The involvement of a DNA intermediate was suggested by Gaidamovich et al. (1978) who reported that LCM gene expression occurred in a mouse lymphoid cell line exposed to DNA extracted from LCM-persistently infected Detroit 6 cells. However, this was not corroborated by Holland et al. (1976) who failed to transfer the LCM genome by transfection. There is no evidence for the presence of a reverse transcriptase activity which would be necessary for the synthesis of an arenaviral DNA intermediate (Carter et al., 1974; Welsh et al., 1975; Scolnick et al., 1970).

Coto and colleagues (1981) have suggested that there is a role for ts mutants which appear in cultures persistently infected with arenaviruses, as has been reported for other virus groups (Preble and Youngner, 1975). The release of infectious virus from Tacaribe-infected Vero cells was found to follow a cyclical pattern in those cells which survived the initial acute infection, with infectivity declining until passage 16 when no infectious virus could be detected in in culture supernatants (Damonte et al., 1981b). The emergence of ts variants was detected from as early as the second cell transfer and by the eighth passage virus produced by the cultures exhibited a plaquing ratio at temperatures of 37° and 40°C in excess of 10^4. This was paralleled by a finding of increased thermolability of virus produced during the course of establishing virus persistence. Interestingly, virus recovered during cell passage did not produce persistent infections when added to fresh uninfected Vero cell cultures, there being complete destruction of the cell sheet as a result of lytic infection, possibly as DI particles are absent from such inocula but present in the virus stocks used to establish the original culture.

Similar findings of ts particle production have also been reported in cultures persistently infected with Junin virus (Damonte and Coto, 1979b). It has been suggested that in this system control of virus persistence is dependent on host cell DNA expression and protein synthesis as the addition of actinomycin D or cycloheximide for up to 24 h results in an increase in viral antigen expression at the cell surface (Coto et al., 1979). These studies also showed that inhibition of protein synthesis within the persistently infected cell resulted in an increase in the re-emergence of infectious virus.

In contrast, there is no evidence of ts mutants in BHK-21 or L-929 cell cultures infected with LCM virus (Welsh and Buchmeier, 1979). This, however, does not exclude the production of other particle types arising in persistently infected cultures in addition to the appearance of DI par-

ticles; for example Popescu and Lehmann-Grube (1976) detected variants which produced turbid plaques after passage of virus through mouse spleens although this virus established persistent infections in newborn mice as readily as virus taken from infected brain tissue which gave characteristically clear plaques *in vitro*.

CHAPTER 9

Immunology of arenavirus infections

Immune responses to arenaviruses are perhaps best understood in acute infection of mice. The outcome depends greatly on both the age of the inoculated animal and the route of infection. Immunocompetent laboratory mice inoculated intracerebrally generally develop a severe disease which is usually fatal. A proportion may survive and become immune, although the exact number is dependent on both the strain of virus and the genetic haplotype of the mice. In contrast, intraperitoneal injection of adults gives rise to an asymptomatic acute infection of 2–3 weeks duration. Buchmeier et al. (1980a) have summarized the responses to LCM virus in immunocompetent mice inoculated intraperitoneally with 10^2–10^5 pfu of virus. During the first two weeks the acute disease may be divided temporally into five phases:

1. a viraemia, reaching a maximum on day 3 and then declining rapidly;
2. almost immediate activation of natural killer cell activity augmented by the simultaneous appearance of interferon to be followed by the generation of activated macrophages 4–5 days later;
3. a marked depression in haemopoietic function (see Chapter 5);
4. development on day 5 of a cytotoxic T-cell response specific for LCM virus-infected cells which reach a maximum by day 9; and
5. the development of immunity as manifested by the appearance of neutralizing antibody from day 4.

Such studies of LCM virus infection in the mouse have resulted in a

number of findings with implications beyond the field of arenavirus research. Firstly, the description by Rowe (1954) of the immune-mediated pathology of acute LCM infection was the first demonstration that the disease potential of viruses may not be related solely to the activity of viruses within infected cells. The role of cytotoxic T cells in lysing LCM virus-infected cells was subsequently explored in other virus systems, and led rapidly to the concept that recognition of a target cell required both the presence of viral antigen and compatibility of the host major histocompatibility complex (Zinkernagel and Doherty, 1979). This restriction phenomenon has had far reaching implications in understanding interactions between T cells and infected cells in many different virus infections. Recent advances in the development of virus-specific T-cell clones are likely to provide further answers as to the nature of this recognition and the degree of interaction with newly synthesized viral proteins (Byrne and Oldstone, 1984). Secondly, the persistence of virus in mice infected shortly after birth has provided a model for understanding both host and viral factors involved in the establishment and maintenance of chronic infection. The finding of virus antigen antibody complexes in persistently infected animals has illustrated that B-cell tolerance is not involved (Oldstone, 1975; Buchmeier and Oldstone, 1978b). Finally, activation of natural killer cell activity early in acute infection coinciding with the production of interferon has helped further our knowledge of innate immunity against virus infection (Welsh and Zinkernagel, 1977; Welsh, 1978).

Several excellent reviews emphasizing work on the immune response to arenaviruses have been published (Buchmeier et al., 1980a; Lehmann-Grube, 1984a) whilst the monograph of Lehmann-Grube (1971) summarizes in detail all of the early work accomplished with LCM virus.

9.1. Interactions with cells of the host-immune system

9.1.1. Lymphocytes

Direct demonstration of arenavirus replication in lymphocytes is of substantial importance for understanding pathogenesis, as these cells provide both a continual source of virus entry into the circulation and play a key role in the temporal and qualitative control of the host-immune response (Murphy and Whitfield, 1975). Tropism of various viruses for cells of the lymphatic system has been reported for various RNA viruses and some viruses are clearly able to discriminate between cells in the

haemopoietic series, e.g. vesicular stomatitis virus will replicate in macrophages but not in lymphocytes or polymorphonuclear leucocytes (Edelman and Wheelock, 1976). However, spleen cells acquire the capacity to replicate this virus when cultured in the presence of Concanavalin A or pokeweed mitogen (Kano et al., 1973).

Studies with LCM virus have shown that cells of the lymphatic system contain viral antigen in persistently infected mice (Mims, 1966; Mims and Wainwright, 1968). Further work (Popescu et al., 1979) has shown that most of the infectious virus in the blood of carrier mice is associated with approximately 2% of the total circulating lymphocyte population. No significant differences between the association of virus and T or B cells could be determined. However, there is some evidence that non-dividing lymphocytes are not susceptible to LCM infection. One possibility is that precursor or immature lymphocytes are susceptible and retain the virus when mature (Popescu et al., 1979). These authors have cited as supporting evidence the work of Brown (1968) who found viral antigen in lymphoid cells both in the blood and thymus of animals infected 12 h after birth. Alternatively, the level of infected cells may be determined by a restricted number of viral receptors either on the surface of all lymphocytes or a fraction of the total lymphocyte population.

Exposure to low levels of virus over prolonged periods would be required for successful infection of a small number of cells. The latter case would require clonal stimulation prior to virus adsorption and replication. Popescu et al. speculate that these cells retain capacity to divide in the presence of non-infectious antigen from other sources similar to the remaining lymphocytes, despite infection with LCM virus. Mouse lymphocytes may support the replication *in vitro* of LCM and other viruses when stimulated to proliferate by addition of phytohaemagglutinin (Schwenk et al., 1971; Eustatia and van der Veen, 1971). Popescu et al. (1979) therefore propose that such clonal expansion may be triggered *in vivo* by viral antigen binding to appropriate lymphocyte receptors. Infection of these cells may then follow which in turn leads to functional inactivation (Cihak and Lehmann-Grube, 1978). Certainly, the growth of many arenaviruses is optimal in actively dividing cells (Pfau et al., 1973; Rawls et al., 1976).

Doyle and Oldstone (1978) have shown that a small population of circulating lymphocytes similarly are infected in acute LCM virus infection showing that infection of peripheral lymphocytes is not related to maintenance of persistent infection *in vivo*. These workers showed that both T- and B-cell populations in addition to macrophages contained virus that could be measured by infectious-centre assay.

9.1.2. Macrophages

Both LCM virus and members of the Tacaribe complex will replicate in macrophages. Peritoneal macrophages cultured *in vitro* support the growth of LCM virus (Mims and Subrahmanyan, 1966) and virus may be recovered from mononuclear cells extracted from blood or infected tissues of persistently infected mice (Doyle and Oldstone, 1978; Popescu et al., 1979). Recovery of virus required the use of an infectious centre type of assay whereby macrophages were co-cultivated with susceptible Vero cells. Importantly, the number of infectious foci was reduced by pretreatment of cells with viral antibody and complement. In the study of Doyle and Oldstone, the number of macrophages inducing infectious centres rarely exceeded the range of 0.1 to 1.0% of total cell number, and the virus replication in mouse macrophages proceeds with no cytopathic effect (Schwarz et al., 1978).

Macrophages from liver, spleen and peritoneum of adult mice acutely infected with LCM virus become activated for the uptake of non-specific antigens within one week (Blanden and Mims, 1973). Buchmeier et al. (1980a) cited unpublished work of R.M. Welsh and M.V. Doyle who found an increase in the number of peritoneal macrophages in acute LCM infection. The cells were found to be heavily vacuolated with up to 40% containing virus-specific antigen up to day 6, but thereafter the number of positive cells declined rapidly, possibly as a result of the elimination of infected macrophages by cytotoxic T-cell activity. These changes are not observed in athymic mice indicating that macrophage involvement may be an event requiring T-cell activity. This has been found for both Sindbis and yellow fever viruses where macrophages play an essential role in controlling virus levels (McFarland, 1974). In this context, susceptibility to flavivirus-induced encephalitis in inbred mouse strains appears to be under the control of a gene unrelated to the MC region (reviewed by Brinton, 1980). For comparison, resistance to lactic dehydrogenase-elevating virus, which only replicates in macrophages, appears to be under the control of two host genes, only one of which may be linked to the H-2 region. This chronic virus infection in mice may be interlinked with host-specific gene expression at the cellular level, particularly cells of the lymphatic system.

Specific viral antigens have been demonstrated in cells of lymphatic and haemopoietic tissues of both humans and guinea pigs infected with Junin virus (Carballal et al., 1977a; Gonzalez et al., 1980) (cf. Chapter 4). Gonzalez et al. (1982) extended these observations further by reporting that both the pathogenic XJ and attenuated XJ-Cl_3 strains replicated

in cultured mouse peritoneal macrophages; however, the attenuated virus gave consistently high titres of virus and in both instances infected cultures survived longer than non-infected cultures. Apart from an increased number of cytoplasmic vacuoles, there was no indication of cytopathic effect. The infected macrophages retained their capacity for phagocytosis, despite indications of having become chronically infected on prolonged culture. Another interesting observation was the finding that peritoneal macrophages from immune animals also supported Junin virus replication but the onset of viral growth was delayed. The possibility that immune interferon or the simultaneous presence of immune T cells become activated by re-exposure to the virus or both was not determined.

9.2. Acute infection

9.2.1. Interferon and natural killer cell activity

There is abundant evidence that type 1 interferon is induced during acute LCM virus infection, reaching maximum titres on day 2–4 (Padnos et al., 1971; Riviere and Bandun, 1977; Bro-Jorgensen and Knudtson, 1977; Merigan et al., 1977). Its appearance correlates with the appearance of infectious virus in the blood and its production at high levels in genetically athymic mice indicates that interferon may be produced directly by infected cells rather than as a result of immune stimulation (Merigan et al., 1977; Welsh, 1978). However, persistently infected animals only show low interferon levels, and the virus does not induce interferon *in vitro* (Welsh and Pfau, 1972).

Interferon is induced both in neonatally infected mice and in adult nude mice inoculated with LCM virus (Gresser et al., 1978; Merigan et al., 1977) and this may be responsible in part for the subsequent development of glomerulonephritis. Buchmeier and Oldstone (1978b) demonstrated that the virus-antibody immune complexes present in the serum of animals chronically infected at birth are deposited in the glomeruli; lesions of the glomerular basement membrane resemble microscopically those induced by infection of exogenous interferon (Morel-Moroger et al., 1978) and may be inhibited by treatment of the mice with anti-interferon antibody.

Transient interferon levels are similarly found in LCM-infected adult nude mice within 7 days although the levels may not necessarily correlate with the absence of a thymus; Merigan et al. (1977) found 8 times higher

levels of interferon in *nu/nu* mice compared to *nu/+* heterozygotes. Similarly, Christoffersen and Bro-Jorgensen (1977) found higher levels of interferon in *nu/+* mice 2 days after infection. However, Ronco et al. (1981) found only similar levels in both homozygous (*nu/nu*) and heterozygote (*nu/+*) animals. Thus different genetic backgrounds may also be important as there is considerable evidence that interferon production is under genetic control (de Maeyer and de Maeyer-Guignard, 1969; Riviere et al., 1980). The level of circulating immune complexes is considerably lower, however, in chronically infected nude animals as compared to neonatally infected mice (Ronco et al., 1981), illustrating that the immune status associated with the development of virus-persistence may differ in athymic animals. Interferon levels *in vivo* may also be responsible for the immunosuppression toward unrelated antigens during acute LCM virus infection (Silberman et al., 1978); this correlates with macrophage dysfunction. Addition of normal macrophages or 2-mercaptoethanol to spleen cells from acutely infected mice restores their proliferative response to mitogen (Jacobs and Cole, 1976).

The appearance of interferon in mice experimentally infected with Junin virus does not seem to play a role in viral pathogenesis. Weissenbacher et al. (1976b) found similar levels of circulating interferon in both infected newborn and adult mice although the virus only produces a disease in suckling animals. Furthermore, newborn mice treated 2 h prior to infection with 50 μg of poly I:C did not significantly alter the outcome of infection, nor if administered continually after virus inoculation, although death was delayed 3 or 4 days in the latter case. Similar results were obtained in infected guinea pigs and confirmed *in vitro* by the finding that no reduction was obtained by induction of interferon in either infected Vero or L cells. Vidal and Coto (1980) found that interferon was produced in a persistently infected cell line prepared by infection of primary mouse embryo fibroblasts. Although heterologous interference could be demonstrated using vesicular stomatitis virus, infectious Junin virus continued to be released in a cyclical fashion on continuous cultivation. Somewhat different findings were reported by Teyssie et al. (1981) who showed that virus replication in the brain of infected animals on day 8 was only marginally reduced by the administration of large amounts of interferon. Lysis of infected target cells by cloned T cell appears not to correlate with gamma-interferon production *in vitro* and furthermore there appears not to be any parallel between interferon production and the extent of cell lysis (Anderson et al., 1985). However, these findings do not exclude a role for interferon production *in vivo* by regulating various arms of the immune response.

Few studies have investigated the levels of interferon in acute arenavirus infection of man. One recent study has shown that elevated levels of interferon can be detected in the early stages of acute Argentinian haemorrhagic fever (Levis et al., 1984). Although high serum levels coincided with the onset of fever and backache, there was no correlation between the titre of interferon and the amount of virus in the blood, although the fall in circulating interferon late in the acute phase occurred at the same time as the appearance of specific antibodies. By analogy to studies observing the effects of exogenous interferon in man, the authors suggested that at least some of the clinical signs may be directly attributable to levels of interferon or indeed some other lymphokine. A depression of platelet and lymphocyte numbers are particular examples. This hypothesis is also in accord with the tropism of Junin virus for both leucocytes and macrophages where interferon secretion is a response to virus replication.

Natural killer cell activity increases with similar kinetics to the appearance of interferon (Welsh and Zinkernagel, 1977). These cells are non-specific in their effect, possessing the capacity to lyse a number of different cell types, are characterized by a lack of surface immunoglobulin, are non-adherent and possess a low concentration of theta antigen (for a review see Welsh, 1981). As early as one day postinfection, spleen cells obtained from infected animals kill both infected and uninfected histocompatible target cells with equal efficiency and show an expanded range of cytotoxic activity against a range of allogenic and xenogeneic cell types. Similar kinetics for the appearance of natural killer cell activity has recently been reported for mice infected with Pichinde virus (Walker et al., 1984). Although the total number of natural killer cells increases, this does not imply division of activated cells as the same effect is seen in mice previously X-irradiated (Welsh, 1978). Once activated, the natural killer cells show enhanced binding to nylon wool columns, enlarged size, and an increase in Fc receptor activity (Welsh and Keissling, 1980a). In LCM infection, splenic natural killer cell activity rapidly decreases until 4 days after infection, almost all of the cytolytic immune cell activity is H-2 restricted (Stitz et al., 1985). However, natural killer cells continue to circulate in the blood until at least 10 days after infection and may mask an underlying specific cytotoxic T-cell response, The role of natural killer cells in controlling arenavirus infection is unclear, however. LCM virus is an excellent inducer of interferon, which may account for the high levels of natural killer cells in spleen and blood. However, evidence that the overall level of LCM virus activity is the same in C57BL/6 mutant beige mice which have markedly reduced numbers of natural killer cells

would mitigate against these non-specific effector cells playing a major part in lysing infected cells early during infection (Welsh and Keissling, 1980b).

9.2.2. Humoral responses

Studies in acutely infected mice have shown that free CF antibody to LCM virus develops after the first week of infection, largely directed against the internal nucleocapsid protein (Smadel and Wall, 1940; Gschwender et al., 1976; Buchmeier et al., 1977), although it should be noted that the larger G1 glycoprotein on the surface of the LCM virion also fixes complement in the presence of specific antibody, although the titres are relatively low (Bruns et al., 1983c). It is unclear as to the role of specific antibody *in vivo*. Although anti-LCM sera lyse infected cells in the presence of complement (Oldstone and Dixon, 1971), depletion of the complement component C3 *in vivo* by administration of cobra venom factor significantly reducing mortality levels in infected mice, the situation is complicated by the lysis of infected cells by complement in the absence of antibody (Welsh and Oldstone, 1977), suggesting that the *in vivo* findings may be explained by a reduction in the numbers of activated macrophages in complement-depleted animals.

Infectious virus-antibody complexes can be detected as early as 4 days after LCM infection of 6-week-old immunocompetent mice (Buchmeier et al., 1980a). The production of IgM antibody appearing in athymic nude mice suggests that induction occurs independent of T-cell activity. By 3 weeks after infection, heterozygous littermates converted to antibody of the IgG glass, whereas only IgM antibody could be detected in athymic animals: these survive the infection owing to the absence of immune-cellular responses directed against virus-infected cells. Further evidence against a role for antibody in the immunopathology of LCM infection is the lack of effect of B-cell depletion; Johnson et al. (1978) found that mice treated with anti-IgM serum from birth succumbed to intracerebral infection as readily as the untreated controls.

In comparison to other virus systems, the numbers of splenic antibody-producing cells releasing anti-LCM antibodies are high, although this may reflect the assay system used (Moskophidis and Lehmann-Grube, 1984). IgM-producing cells appear as early as 4 days after intravenous inoculation and an IgG response 2 days later, although the numbers of IgG-producing cells appear considerably fewer in persistently infected animals. In immune mice, IgG2a subclass antibodies specific for the viral nucleocapsid have been detected (Randrup Thomsen et al., 1985). As

B-cell responses including isotype expression are under T-cell control, T-cell priming by individual antigens on the nucleoprotein may arguably be more effective than by antigens on the surface glycoproteins of the virus.

Immunity to arenaviruses appears generally to be type-specific; an infection with one member of the family does not necessarily confer protective humoral or cellular immunity for arenaviruses serologically unrelated by the neutralization test. However, study of certain virus pairings have shown that cross-reactive antibodies may confer some degree of protection. For example, immunization of experimental animals with Tacaribe virus protects against subsequent challenge with the normally virulent Junin virus, and varying levels of protection may be conferred by prior immunization with less closely related viruses, e.g. Amapari virus (Weissenbacher et al., 1975/1976). These responses are clearly distinguished from anamnestic responses that may be observed as a result of antigenic similarities between the nucleocapsid proteins of otherwise unrelated arenaviruses. For example, the possibility that serological similarities between LCM and Junin viruses could be sufficient to allow some kind of anamnestic response to Junin virus, despite the lack of cross-reactivity in the CF test, has been considered in experimental animals sequentially infected with Junin and LCM viruses (Barrera Oro, 1977). Guinea pigs inoculated first with the attenuated XJ-Cl_3 strain of Junin were challenged with 300 LD_{50} of the Armstrong strain of LCM virus approximately 4 months later. As had previously been seen in some human cases of Argentinian haemorrhagic fever, CF antibodies against both Junin and LCM viruses rose simultaneously after the LCM virus challenge. However, neutralizing antibody levels against Junin were elevated prior to the appearance of specific neutralizing antibody to LCM virus. This serological relationship by the CF test almost certainly reflects closely identical protein sequences on the internal nucleocapsid. In contrast, neutralizing antibody is directed against the unique polypeptides present in the outer envelope of these viruses, thus explaining the absence of a secondary neutralizing antibody response.

9.2.3. Cell-mediated immunity

The role of cell-mediated immunity during acute LCM infection is manifested by a cytotoxic T-cell response associated with the clearing of virus (Zinkernagel and Welsh, 1976). This has unequivocably been confirmed by the recent findings of Byrne and Oldstone (1974) that T cells cultured and cloned *in vitro* cleared at least 2 logs of virus from the spleens of acutely infected mice when administered intravenously. This adoptive

transfer effect using cloned T cells appeared to last over several days following a single inoculation. The restriction of the cytotoxic T-cell response to dual recognition of both viral and host cell proteins encoded by the H-2 region has profoundly altered our concept as to how the infected host clears virus from infected tissue and has been described in every virus system examined (reviewed in detail by Zinkernagel and Doherty, 1979).

The background of this work was the report by Oldstone et al. (1973) that susceptibility to LCM virus differed among various inbred mouse strains representing a range of haplotype. The overwhelming evidence that the fatal disease in adult mice was caused by a specific T-cell response suggested that disease severity in different mouse strains may be related to the extent of this immunopathology. Following this hypothesis, Zinkernagel and Doherty injected intracerebrally various H-2 haplotypes with the Armstrong strain of LCM virus and 7 days later the cytotoxicity of spleen cells examined on infected L cells, a mouse fibroblast line originally derived from C3H mice. Only spleen cells from mice of the same haplotype ($H\text{-}2^k$) as the C3H-derived fibroblast line were found to lyse the infected target cells; intermediate levels of cell lysis were obtained using cytotoxic T-cell preparations from mice compatible only in either the K or D region of the major histocompatibility complex (Zinkernagel and Doherty, 1974; Zinkernagel and Welsh, 1976). There is abundant evidence that such H-2 restriction is at the level of cytotoxic T-cell generation and not merely in the effector phase (Zinkernagel and Doherty, 1979); although compatibility in the K and D regions appears equally important, recent work in influenza-infected mice has shown that the route of inoculation may lead to a more defined restriction at the D locus if virus is inoculated intranasally rather than intraperitoneally, presumably as the antigen presenting cells differ locally in capacity to effect T cell co-operation necessary for inducing a cytotoxic response (Pala and Askonas, 1985).

The generation of specific cellular toxicity is inter-related to the replication of the virus in target organs (Buchmeier et al., 1980a). Inoculation with live virus appears necessary as a primary cytotoxic T-cell response is not seen if the virus is first inactivated (Zinkernagel et al., 1978). This may say something as to the method of virus entry into susceptible cells, as generally an immune response can be induced by non-infectious virus that remains capable of fusion at cell membranes (Schrader and Edelman, 1977) and have implications for the development of inactivated arenavirus vaccines should the stimulation of cellular immunity be essential for protection. The generation of primary *in vitro* responses has

proved very difficult, thus hampering the necessary experimental studies to clarify the nature of viral antigen presentation involved in stimulating cellular immune responses. These *in vitro* responses tend to be at a low level and highly variable (Dunlop and Blanden, 1977b).

The answers to many questions regarding the nature and fine specificity of the cytotoxic T-cell response are likely to come from the study of T-cell clones selected and cultured *in vitro*. Oldstone and colleagues have recently reported a series of experiments using T-cell clones produced from mice infected with the Armstrong strain of LCM. Spleen cells were obtained from H-2^b animals 4–8 weeks after virus inoculation and stimulated *in vitro* by co-cultivation with infected syngeneic macrophages prior to cloning by limiting dilution. A total of 12 clones specific for LCM infected target cells were obtained, all of which were restricted to targets of haplotype H-2^b (Byrne et al., 1984). Neither of these clones were found to lyse cells infected with Pichinde virus, although all recognized H-2 restricted target cells infected with a wide range of different LCM virus strains known to cause a wide range of disease in inbred mice. This cross-reactivity was observed regardless of the fine specificity of the H-2^b restriction as some clones were restricted to the K region whereas the remainder recognized the D end of the H-2 region. Furthermore, this cross-reactivity was seen equally in clones recognizing different epitopes within the K region. H-2 restricted targets infected with the homologous Armstrong virus and the Traub and WE strains were lysed whereas cells infected with the Pasteur and UBC strains remained viable. This broad cross-reactivity for conserved viral determinants may not be universal to all possible cytotoxic T-cell clones, however, as one line from an H-2^d mouse clearly distinguished cells infected with different strains. Adoptive transfer experiments have shown that virus titres in acutely infected mice are reduced substantially by a single intravenous injection of cloned T cells and that this reduction is genetically restricted (Byrne and Oldstone, 1984).

The molecular basis of T-cell-target cell interactions remain to be fully resolved, although it is known that the host cell H-2K and D epitopes reside on a membrane structure consisting of a 45 000 molecular weight glycoprotein non-covalently bound to the 12 000 molecular weight polypeptide beta-2 microglobulin (Coligan et al., 1981). A comparison of H-$2K^b$ and H-$2K^d$ gene sequences has shown that polymorphism is generated only by the introduction of nucleotide changes in clusters equivalent to 3 to 8 amino acids in the expressed molecule; restriction mapping suggests that the H-$2D^b$ and H-$2D^d$ regions are quite different (Weiss et al., 1984). The cytotoxic T-cell clones specific for LCM virus developed

by Byrne and Oldstone (1984) appeared to recognize multiple epitopes in different domains of the H-2K^b molecule, but whether recognition truly involves conformationally dependent determinants on the 48 000 molecular weight glycoprotein in the manner that has been suggested for immune T cells directed against vaccinia-infected targets remains to be determined. One unexplained finding is the remarkably high efficiency of this process *in vivo* (Lehmann-Grube et al., 1985). The rate of virus elimination appears to be greatest at the time when cytotoxic T cells become detectable and adoptive transfer experiments show that few T cells are required to limit virus replication. Possible lymphokine secretion to supplement direct cytolytic activity *in vivo* may account for this.

As to which virus-specific determinants are recognized by LCM specific cytotoxic T cells has yet to be determined. Byrne and Oldstone (1984) have noted that the larger envelope glycoprotein G1 is by far the predominant viral polypeptide expressed on the surface of infected target cells with the second glycoprotein G2 and the common precursor molecule constituting the remainder (Buchmeier et al., 1978; Buchmeier and Oldstone, 1979). Whether these represent the viral contribution to the receptor site for T-cell recognition is not clear, although at least several unique viral epitopes are recognized by cytotoxic T-cell clones, several of which are shared by different LCM strains. One surprise from the study of influenza virus T-cell clones specific for different viral subtypes has been the discovery that immune cell recognition does not depend on the presence of viral envelope glycoproteins of the particular virus subtype (Townsend and Skehel, 1982). Whether this will turn out to be the case in active cellular immunity against LCM virus infection will be of great interest. Oldstone and colleagues point to the fact that, unlike the influenzavirus system, LCM virus is a natural pathogen of mice and the cellular immune system may display a different spectrum of specificity for viral determinants. One approach to understanding the specificity of T-cell clones is to analyze the degree of inhibition of virus release *in vitro* and to correlate this with the expression of viral polypeptides within and at the surface of the infected cell. Cloned cytotoxic T cells clearly inhibit the release of infectious virus from histocompatible cells as late as 12 h after infection and prior to the expression of viral antigen within the cytoplasm or at the cell surface (Anderson et al., 1985). The physical interaction between the effector and target cell showed extensive convolution of the infected cell with a number of finger-like protrusions of the T cell penetrating into the cytoplasm of the infected cell. Inhibition of viral glycoprotein transport to the cell surface did not prevent target cell lysis, although no cytotoxicity could be shown using T cells from the spleens of infected

animals on such cells. Whether this result reflects the varying stages of differentiation between splenocytes and cloned cells is not clear but we may now expect rapid advances in this area of understanding as to just exactly how cytotoxic T cells aid recovery from virus infection.

There is some considerable variation in the degree of cytotoxic T-cell activity according to the strain of mouse selected. Moskophidis and Lehmann-Grube (1983) examined a total of 14 strains and found a spectrum of cytotoxicity responses which did not correlate with the level of virus replication. High and low responders were manifested by DBA ($H\text{-}2^q$) and CBA ($H\text{-}2^k$) strains respectively, although it was noted that there were significant differences between mice of identical haplotype. In general, there was a correlation between the level of measurable cytotoxic T-cell activity and specific footpad responses reported earlier from the same laboratory (Lehmann-Grube and Lohler, 1981). There appears, however, to be little difference between inbred strains in terms of humoral antibody production (Kimmig and Lehmann-Grube, 1979).

The extent of cytotoxic T-cell activity may also vary according to the dose of virus, being higher when the inoculum contains a low titre but lower when the infectious inoculum is increased (Doherty et al., 1974; Dunlop and Blanden, 1977; Cihak and Lehmann-Grube, 1978). The normally lethal effect of LCM virus inoculated intracerebrally can be abrogated by inoculation of 10^7 infectious units or higher of the E-350 strain into CBA/J ($H\text{-}2^k$) mice (Lehmann-Grube et al., 1981). This also led to a reduction in delayed type hypersensitivity reactions. Transfer of spleen cells together with virus up to 3 months after infection into irradiated recipients resulted in a rapid cytotoxic T-cell response. Taken together, these observations point to active and long-lasting suppression of normally lethal cytotoxic T-cell activity if the artificial condition of high virus dose is adopted. There appear to be differences between virus strains, however, as the use of the WE strain of LCM virus resulted in a much reduced difference in cytotoxic T-cell response between animals receiving high and low titres of virus. Secondary cell-mediated responses could be clearly demonstrated on subsequent challenge in animals infected previously with the WE strain, in clear contrast to the absence of secondary responses in mice previously inoculated with a high dose of E-350 virus. Secondary cell-mediated responses could not be demonstrated on subsequent challenge in animals infected previously with a high dose of E-350 virus. In contrast, secondary responses were clearly seen in mice previously exposed to the WE strain, despite the presence of neutralizing antibody.

In a recent paper, an attempt has been made to demonstrate secondary

responses to Pichinde virus in a mouse model system (Walker et al., 1984). A strong cytolytic T-cell response was demonstrated against virus-infected syngeneic target cells using splenocytes from infected C57BL/6 (H-2^b) mice, although a variety of inbred mouse strains were found to respond to the virus. Secondary infection of mice with Pichinde virus resulted in a more rapid appearance of cytolytic responses, and this included a virus-induced natural killer cell response in addition to H-2 restricted cytotoxic T-cell activity. These secondary responses appeared to depend less on cell division as compared to the primary response when measured by sensitivity to cyclophosphamide.

Parodi et al. (1967, 1970a) demonstrated that Junin-infected guinea pigs had a decreased primary and secondary response to sheep erythrocytes as measured by both circulating antibody levels and the number of antibody-forming cells obtained from popliteal lymph nodes. The inhibitory effect on the primary response was abrogated in animals passively immunized with homologous antibody, although the secondary response was unchanged (Carballal and Frigerio, 1973), suggesting that the immunological memory was not impaired during infection.

9.3. Chronic persistent infection

9.3.1. Detection of B-cell responses

Although early attempts to demonstrate the presence of circulating antibody in the sera of persistently infected mice were unsuccessful, Oldstone and Dixon in a series of studies clearly proved that carrier animals produced a specific humoral response to viral antigens (Oldstone and Dixon, 1969, 1970b; Oldstone, 1975). This finding was contrary to the previously held view that viral persistence was established or maintained *in vivo* as a result of an absence of specific B-cell responses to some or all viral antigens which would otherwise lead to elimination of the virus. As viral proteins continue to be produced in the tissues of such animals, circulating antigen-antibody complexes are formed which can be detected by binding to Clq (Oldstone et al., 1980). The levels of specific antibody were found to approximate those present in the sera from hyperimmunized animals and deposition of immune complexes occurred in arteries, choroid plexi and renal glomeruli. Antibody was recovered from the kidneys of carrier mice by low pH treatment of tissue slices and its reactivity confirmed both by binding to virus-infected cell cultures (Oldstone and Dixon, 1975) and by direct precipitation of LCM viral polypeptides

(Buchmeier and Oldstone, 1978b). An important piece of evidence was the finding that both sera and kidney eluates from persistently infected mice contained antibodies to all the major structural polypeptides, thus indicating that failure to eliminate virus *in vivo* was not due to a selected deficiency in antibody response to a specific protein. These studies do not preclude, however, that antibody to selected epitopes was reduced or lacking in these animals as compared to hyperimmune sera.

The severity of immune-complex disease appears to be related to the strain of persistently infected mouse. When compared with antibody levels, a positive correlation was found between specific IgG and disease (Oldstone and Dixon, 1970). The levels of Clq-binding immune complexes in the sera of SWR/J (H-2^q) mice infected soon after birth peaks at 8 weeks and thereafter rapidly declines. These mice produce approximately 4 times as much antibody compared to the BALB/WEHI (H-2^d) strain, in which little Clq-binding complexes are found and which have a correspondingly milder glomerulonephritis (Oldstone et al., 1980). In contrast, animals of either strain immunized as adults produced equivalent amounts of antibody.

Production of viral antibody in persistently infected mice is thymus-dependent (Oldstone et al., 1980). Athymic nude mice persistently infected with LCM virus at birth failed to generate circulating antibody. However, *nu*/+ littermates with the same genetic background and similarly infected at birth clearly showed specific antibody by 4 months of age. Immunoprecipitation studies showed that the specificity of antibodies in such sera to be the same as that of antibody eluted from the kidneys of carrier mice. It is worth noting that, despite the existence of antibody to all LCM viral structural polypeptides, sera from persistently infected mice are negative by CF methods, the original basis for believing that carrier animals did not produce a humoral response to the virus. Although it is now known that antibodies are indeed a feature of persistent infections, the possibility still exists that a non-complement-fixing subclass of IgG is selectively induced in the carrier state. However, there is no data as yet which specifically addresses this question. Likewise, low titres or negative findings of neutralizing antibody in the sera of persistently infected mice has yet to be examined in depth. Buchmeier and colleagues (quoted in Buchmeier et al., 1980a) found that antibody in such sera would bind to the surface of virus-infected cells, but did not mediate complement-dependent cytolysis, suggesting that viral antigens at the plasma membrane may either be masked thereby preventing further immune reactions or induce their removal by a process of antigenic modulation.

Little is known as to the mechanism leading to depression of B-cell responses in mice persistently infected with LCM virus; one possibility is the continuing production of viral antigen consumes at a steady rate the amount of circulating antibody produced in response to the chronic infection. Alternatively, the level of antibody production may be depressed, a possibility given the recent report by Moskophidis and Lehmann-Grube (1984) that the numbers of IgG-producing cells in the spleen are much reduced in chronically infected mice.

Different states of T-cell priming toward viral antigens may result in a qualitative difference in antibody specificity in persistently infected mice, a possibility suggested by the studies of Randrup Thomsen et al. (1985) who have found considerably reduced levels of IgG2a antibody in C3H ($H\text{-}2^k$) mice infected at birth. This inbred strain shows a much reduced level of immune complex disease, presumably as a result of lower amounts of virus-specific antibody which activate complement.

9.3.2. Role of cellular immunity in maintaining persistent infections

Whilst the presence of antibody in persistently infected animals implies active B and helper T-cell responses, cytotoxic cellular immunity has not been consistently demonstrated (references summarized in Buchmeier et al., 1980a; Lehmann-Grube et al., 1983). It has been difficult, therefore, to distinguish between suppression of T-cell activity and complete absence of cytotoxic T-cell clones responsive to some or all of the relevant LCM viral antigens expressed *in vivo* on the surface of infected cells. However, it is widely recognized that carrier mice respond normally to unrelated immunogens (Oldstone et al., 1973; Guttler et al., 1975). Thus, in this respect these animals show immunological tolerance to the virus. Here it is pertinent to mention that persistence of LCM virus in mice infected either at birth or *in utero* was one of the important observations made by Burnet and Fenner (1949) to support the concept of tolerance to 'self' antigens. Although this general concept of clonal selection is still applicable to a discussion of LCM persistence, the findings of a thymus-dependent antibody response in persistently infected mice would restrict this deletion to cytotoxic T cells, clones of T-helper cells remaining intact regardless of the disease state in these animals. The time of infection appears critical as LCM infection induced 24 h or later after birth results in the generation of a cytotoxic T-cell response typical of acute disease (Cihak and Lehmann-Grube, 1978). The failure of mice infected prior to this time to mount an adequate cytotoxic response is presumably related to maturation of T-cell function and appears to be virus-specific as adult

carrier mice challenged with other unrelated arenaviruses show normal cytotoxic T-cell responses (Buchmeier et al., 1980a). Thus the block appears to be either in the recognition or expression of cells recognizing, type-specific antigenic determinants, presumably on viral structures normally expressed on the surface of infected cells, or both.

An alternative is the active suppression of certain type-specific cytotoxic T-cell responses and there is some evidence to favour this possibility. Zinkernagel and Doherty (1974) found that LCM virus-specific T-cell cytotoxicity could be induced 9 days after the adoptive transfer of allogenic spleen cells into adult mice carrying the virus, thereby effectively interrupting active suppression of the cytotoxic response. Dunlop and Blanden (1977a) also showed that cytotoxic T-cell activity during acute LCM infection could be suppressed by a variety of ways, including the addition of virus or infected cells into the assay system.

Reports from several laboratories that a small proportion of circulating lymphocytes are infected in persistently infected animals (Popescu et al., 1977, 1979; Doyle and Oldstone, 1978) has led Lehmann-Grube and colleagues to propose that lymphocytes with the potential of responding to the virus are functionally inactivated if they react in the first instance with infectious virus (summarized in Lehmann-Grube et al., 1983). This hypothesis assumes a variation in the role of macrophages or other antigen-presenting cells according to the age of the animal. Prior to birth and during the first 24 h such cells support virus replication and release infectious virus in the close proximity of other cells of the maturing lymphoid system. Later in life, however, the suggestion is that macrophages handle the virus in a different manner and present on their surface essentially non-infectious viral antigens. Certainly, there appears to be an age-dependent sensitivity of circulating T lymphocytes to virus infection. Tijerina et al. (1980) showed that the number of T cells scoring as positive infectious centres decreased markedly according to the inoculation time after birth, with somewhat greater sensitivity of cells obtained from the thymus during the first 3 days of life. In all instances, however, the total percentage of cells scored as positive for virus never exceeded 0.01%, although this may be an underestimate owing to the relative insensitivity of the *in vitro* assay procedure employed. It should be borne in mind that these experiments also measure susceptibility of mature T cells expressing Thy.2 antigen only and do not account for the susceptibility of other lymphoid cells. The peak in numbers from cells obtained from animals infected at less than 24 h after birth was 5 days later in cells from the thymus although splenic lymphocytes continued to produce virus for extended periods (Lehmann-Grube et al., 1983). It is difficult to conclude

whether these experiments identify a relevant subset of maturing lymphocytes from the point of view of analyzing the lack of cytotoxic T-cell responsiveness in mice infected as newborns, however, although the decline over the first few days of life coincides with the proportion of animals which fail to eliminate the virus when inoculated at successively later times after birth. It is not totally clear as to the corresponding level of replication in the macrophages at this time, although preliminary observations from Holscher and Lehmann-Grube (cited in Lehmann-Grube et al., 1983) suggest that macrophages in newborn animals support virus replication at lower levels compared to cells obtained from adults.

The ability of persistently infected animals to generate virus-specific antibodies mediated by T cells raises the question of a possible role for suppressor T cells. However, the identification of suppressor cells has not been successful (Cihak and Lehmann-Grube, 1978). Antibody feedback may be a control mechanism of importance in the carrier state but the variation in antibody levels between inbred strains of mice makes this unlikely. Clearly, the linkage between helper T-cell activity and B-cell responses is difficult to define in the carrier state and there is a distinct lack of data to clarify this point. Several observations indicate that suppression of cytotoxic T-cell activity and antibody levels often are independently controlled. For example, inoculation of adults with large amounts of virus results in a significant number becoming persistently infected without any appreciable change in the level and type of circulating antibody (Hannover and Larsen, 1968). Simultaneous inoculation of virus and syngeneic lymphocytes from uninfected adults into susceptible newborns also gives the same result (Bro-Jorgensen and Volkert, 1974). In any event, it should be remembered that there are several alternative pathways by which B cells may be activated and that antigen load, conformational state of viral epitopes presented to the immune system and the degree of involvement of non-specific stimulatory mediators may all play a part and compensate for any deficiency in a particular pathway of antibody generation that becomes blocked as a result of viral infection.

Although infection of adult mice with LCM virus invariably gives rise to a lethal acute disease, there is a marked difference in the proportion of survivors in certain combinations of virus strains and mice of different H-2 haplotypes and such variations make the comparison of results between different laboratories difficult to interpret. For example, the Armstrong (CA-1371) and E-350 strains of LCM virus cause uniformly lethal infections in newborn C3H ($H\text{-}2^k$) mice (Doyle et al., 1980; Dutko and Oldstone, 1983) but survival rates in similarly infected newborn

BALB/WEHI mice were 87% and 95%, respectively (Dutko and Oldstone, 1983). These survivors became persistently infected with the virus. In contrast, WE and Traub strains readily induced persistent infections in both inbred strains. Two further strains, the UBC and Pasteur viruses were intermediate in terms of causing death in approximately two-thirds of C3H mice. Corresponding analysis of RNA segments from these different strains have also been described (see Chapter 7).

In contrast to LCM virus, little information is available concerning immunity in rodents persistently infected with other arenaviruses. Limited studies have been reported using Tacaribe virus and other serologically related members of the Tacaribe complex. With the exception of Latino virus, all members of this group induce a fatal infection of newborn mice (see Chapter 5). However, it should be recognized that careful comparative studies using inbred mice strains have yet to be reported. Almost no information is available concerning the immune response to Lassa and related viruses in the murine system. The normally observed lethality of Tacaribe virus for newborn mice can be abrogated by passive administration of horse anti-mouse lymphocyte serum (Borden et al., 1971) with the establishment of persistent virus infection. There was no evidence of CF antibody in the sera from mice carrying Tacaribe virus, although application of more sensitive antibody detection methods would be required to re-examine this point. Although neutralizing antibody to Tacaribe virus can be readily detected in immune sera, there was no evidence of neutralizing activity in the sera of animals persistently infected in the study of Borden et al. Similar results have also been obtained in thymectomized mice infected both with Tacaribe and other related viruses (Parodi et al., 1970; Besuschio et al., 1973). A curious exception in the latter study was the apparent absence of survivors in thymectomized mice infected with Amapari virus. More recent studies with athymic nude mice and heterozygous (*nu*/+) littermates has confirmed the development of persistent Junin infection in the absence of an effective T-cell response (Weissenbacher et al., 1983). Again, virus was consistently recovered from the brain and circulation of these animals and no neutralizing antibody could be detected. A similar lack of neutralizing antibody is seen in animals persistently infected with Machupo virus. Justines and Johnson (1969) successfully established a colony of *Calomys callosus*, the natural rodent reservoir of this human pathogen. Newborn mice infected before the age of 3 days developed a viraemia which continued in excess of one year of observation. The uniformly high rate of persistence was also seen in animals infected 10 days after birth, but adults showed a split response. Approximately half continued to

develop persistent infections in the absence of neutralizing antibody whereas the remainder mounted an effective neutralizing antibody response to the virus and virus titres in infected tissues began to decline. One interesting observation in this study was that virus took many months to be eliminated completely, despite the presence of specific antibody, perhaps indicating that these animals were still unable to mount a totally effective immune response to the virus.

CHAPTER 10

Treatment and prevention of human infections

Several approaches towards the treatment of human arenavirus infection have been proposed, perhaps the most successful being the use of plasma taken from individuals convalescing from infection. Many questions remain to be resolved, for example, just how effective is such treatment when administered to individuals acutely ill with a virus strain of different geographical origin to that which originally infected the donor. Assessment of viraemia levels in the patient together with an accurate determination of neutralizing antibody levels in immune plasma are emerging as the key factors in assessing the efficacy of such treatment. Antiviral therapy appears to be of more restricted use, although there may be some advantage in the combined use of immune plasma and ribavirin in patients acutely ill with Lassa virus.

Passive immunization has yet to be investigated thoroughly. Lack of suitable starting material is one factor, although the use of immunoglobulin from seroconverted primates may be of use here if doubts concerning cross-reactivity between human and monkey antibody molecules could be investigated. Active immunization to date has only been achieved against Junin virus although there are good prospects for attenuated or inactivated virus vaccines against other arenavirus infections, particularly Lassa fever as more information is rapidly accumulated as to the basic properties of these viruses. It is worth noting that attempts to actively immunize against Argentine haemorrhagic fever represents to date the only successful prophylactic trial against a severe haemorrhagic

fever in man. This experience should prove valuable for the preparation of vaccines not only against other human arenavirus pathogens but also other agents, for example, the filoviruses Marburg and Ebola.

The particularly close relationships revealed by animal studies between arenaviruses non-pathogenic and pathogenic for man are intriguing and the possibility that such viruses may offer one approach to the development of live vaccines has yet to be definitely excluded. Further developments in the field of gene cloning may also offer a future alternative to the conventional means of attenuating or killing homologous virus preparations, although safety considerations concerning the growth of large virus stocks prior to inactivation and possible reversion of attenuated strains pose real problems. Select expression of relevant genes cloned into *E.coli* or yeast plasmids would offer a way round these problems. Whatever the final approach, the number of individuals at risk is certainly too small to attract the interest of commercial vaccine manufacturers. Reliance on national institutions is thus the most likely outcome for the exploitation and development of any successful research findings.

10.1. Antiviral compounds

Several antiviral licenced agents for use in man are known to inhibit *in vitro* replication of arenaviruses. For example, the addition of amantadine hydrochloride to cell monolayers infected with LCM or members of the Tacaribe complex reduces the yield of virus (Coto et al., 1969; Welsh et al., 1971; Pfau et al., 1975). The drug appears to delay penetration of virus into cells and, in contrast to other virus systems sensitive to amantadine, continues to depress yields if added at any point in the replication cycle. This latter effect appears specific for arenaviruses and points to an inhibition in the release of newly formed virus particles at the plasma membrane of infected cells. The drug does not directly inactivate extracellular virus nor prevent adsorption of virus to cells. Transposing these results to measuring the efficacy *in vivo* has not been attempted, however. One problem may be that the addition of amantadine to infected cells at high concentrations rarely decreases virus yields by more than 1–2 logs, thereby questioning whether its use in animal or man would significantly reduce viraemia and the levels of virus in infected tissues.

Isatin beta-thiosemicarbazones are known to inactivate or inhibit the replication of a wide number of DNA and RNA viruses (Bauer, 1977). LCM and other arenaviruses are directly inactivated by isatin betathio-

semicabarzone in the presence of divalent metal ions, presumably as a result of direct penetration of the viral envelope and binding to the nucleic acid genome or the virion-associated RNA polymerase (Logan et al., 1975). Related heterocyclic compounds have been shown to have a more direct effect on virus replication. Pfau and Camyre (1968) found that LCM production in HeLa cells was inhibited by up to 90% by 2 (alpha-hydroxybenzyl)-benzimadazole. Greater potency has been reported for a derivative of this compound, provided the cells were exposed to the drug at least 8 h prior to infection and that mouse L cells were used as the substrate (Stella et al., 1974a).

This derivative has also been found to have an effect *in vivo*; mice receiving the compound in their drinking water were found to survive for at least 4 times as long as untreated control animals infected with LCM virus (Stella et al., 1974b). However, all eventually succumbed to the infection with clinical signs typically associated with LCM disease. In the absence of any effect on the titre of virus present in infected organs during treatment, the prolonged survival was supposed to have resulted from immunosuppression, although this hypothesis was not tested.

Several studies have indicated that the antiviral drug ribavirin (1-B-D-ribofuranosyl-1, 2, 4-triazole-3-carboxamide) may be useful in the treatment of acute arenavirus disease. This drug, also known as Virazole, is a synthetic nucleoside analogue with a broad spectrum of anti-viral activity against viruses with either RNA or DNA genomes and may be administered orally to patients in a dose of 800 mg daily for a month without evidence of toxicity (Muller, 1979; Sidwell et al., 1972). Ribavirin inhibits *in vitro* replication of Lassa virus in both Vero cells and alveolar macrophages (Jahrling et al., 1980). The degree of inhibition appears dependent on both cell type and multiplicity of infection, however, as negligible effect was seen at a concentration of 50 ng/ml in Vero cell cultures at multiplicities above 1 pfu per cell. In contrast, no replication was seen in macrophages infected over a wide range of multiplicities at a much lower ribavirin concentration of 10 ng/ml. Inhibition of virus replication correlated with a reduction in the amount of viral antigen within infected cells.

Rhesus monkeys infected with Lassa virus have been successfully treated with ribavirin (Jahrling et al., 1980). Animals were injected intramuscularly with 50 ng/kg either on the day of inoculation or after 5 days followed by regular doses of 10 ng/kg thrice daily. All the animals survived with a moderation of clinical symptoms and reduced viraemia. The treatment regimen appeared to have little effect on the appearance of viral antibody as compared to untreated control animals, indicating

that immunosuppression did not result from the therapy. Extension of these experiments to the treatment of Lassa fever in man has been initiated although early attempts to treat acutely ill patients in West Africa have proved disappointing (McCormick et al., 1980). This may in part be due to the dose level which at 15 ng/kg day was much lower than that adopted by Jahrling and colleagues for the treatment of infected monkeys. One problem in the use of higher dose levels is the potential effect on the red blood cell count (Togo and McCracken, 1976). Treatment of otherwise healthy monkeys on a regimen consisting of one dose of 33 ng/kg and successive doses of 68 ng/kg per day and 23 ng/kg per day for 4 and 6 days, respectively resulted in anaemia, although haematocrit and haemoglobin levels quickly returned to normal soon after treatment ceased (Wannarka et al., 1982). However, this side effect may be acceptable given the potentially life-threatening situation in acutely ill Lassa patients.

10.2. Passive immunization: the use of immunoglobulin

The use of non-human primates in studying the pathogenesis of haemorrhagic fevers has been extended to the evaluation of passive prophylaxis using immune plasma of human origin. Eddy et al. (1975b) have described one study using an immunoglobulin preparation obtained by Cohn fractionation of pooled sera obtained from 14 cases of Bolivian haemorrhagic fever. All had histories of prior Machupo virus infection and contained neutralizing antibodies against the virus with a dilution endpoint of 1:128. After purification, the final immunoglobulin preparation had a titre of 1:2048 and a protein concentration of 167 mg/ml. Groups of seronegative rhesus or cynomolgus monkeys were treated with varying concentrations of immunoglobulin 4 h after challenge with 1000 pfu of Machupo virus. Significant protection occurred in animals receiving 0.5 ml or more of immunoglobulin per kilogramme body weight. All animals in these groups survived. In contrast, all animals challenged with virus in the absence of antibody died by day 16. Although the subsequent development of antibody among the treated animals was variable, all were resistant to further challenge 56 days after the initial exposure to virus and exhibited an anamnestic antibody response. The administration of immunoglobulin 4 h before the initial challenge resulted in a much slower decline in passively acquired antibody levels.

Neutralizing antibody was still found in the sera of these animals 28 days later. In contrast, antibody was barely detected by 21 days in mon-

keys receiving immunoglobulin after the virus challenge.

An important finding by Eddy and colleagues was the development of late neurological symptoms in passively protected animals. This was manifested as an encephalitis without haemorrhagic signs with an abrupt onset and invariably proved fatal within 4 to 6 days. No viral antigen could be detected by immunofluorescence in the tissues of these animals although this syndrome may be related to the spontaneous encephalitis occasionally seen in acutely infected primates, it is interesting that the passively administered antibody levels in these animals had become undetectable prior to the onset of the syndrome. Some immunological imbalance may be implied by these findings, but at the present time it is unclear if either antigen-antibody complexes play any role in late development of encephalitis or some unrelated viral factor is responsible.

The first use of immune plasma in the treatment of an acute Lassa infection was described by Leifer et al. (1970). A 58-year-old arbovirologist became ill whilst studying animals infected with clinical specimens from a case of Lassa fever. The patient was admitted to hospital 6 days after the onset of symptoms, and 4 days later became the cause of considerable concern. As a result, 500 ml of immune plasma was administered. The source was a missionary nurse who was transferred from Nigeria to New York for treatment of acute infection 4 months previously (Frame et al., 1970). Leifer et al. (1970) described the plasma as having a CF titre in excess of 1:32 and neutralized at least 100 $TCID_{50}$ of infectious virus. Despite a high titre of specific antibody, the masking of residual infectious virus was a prime consideration, particularly in the diagnosis of Lassa fever if the patient had not been confirmed at that time. The patient began to rapidly improve within 24 h of receiving the plasma, becoming afebrile 7 days later. Although laboratory diagnosis of Lassa fever infection was confirmed retrospectively by direct isolation of virus from blood samples taken from the patient immediately after admission, no virus was isolated from serum subsequent to the giving of convalescent plasma; however, virus continued to be isolated from throat washings for at least 2 weeks and in urine for over a month. This illustrates that particular care must be taken in the monitoring for virus in body fluids for some considerable time after a patient enters convalescence, even if immune plasma has been used to treat the patient. In this instance, CF antibody was not detected in significant amounts until the 52nd day after the onset of illness.

Monath et al. (1974b) reported the use of immune plasma in two acutely infected patients admitted to the Panguma Catholic Hospital, Zorzor, during the 1972 outbreak in Sierra Leone. Both patients received 250 ml

of plasma drawn from the same nurse who previously donated blood for the treatment of the patient in New York. The plasma had been taken approximately 9 months after the onset of illness and stored at –20°C prior to use. The first case was a 20-year-old woman admitted approximately 14 days after the onset of symptoms. At the time of administration, the oral temperature was 101.2°F which declines to 98.6°F after 12 h and was accompanied by both subjective and clinical improvement. During the next 48 h, the patient's condition continued to improve with the disappearance of headache, vomiting and dizziness. A similar improvement was seen in the second patient, a 35-year-old woman in the second week of illness. The patient became afebrile and symptoms cleared by 72 h after treatment with immune plasma. In both cases, there was a marked improvement in the respective leucocyte counts over the same period. However, virus was still present in the pharynx of the second patient 8 days after transfusion, again illustrating that virus activity may continue in convalescent patients despite the development of circulating antibodies.

Definitive conclusions as to the benefit of administering immune plasma to patients with Lassa fever, however, are complicated as the disease is frequently self-limiting with rapid clinical improvement in the 2nd or 3rd week of illness. A more objective study as to the efficacy of immune plasma in the treatment of human arenavirus infections is that of Maiztegui et al. (1979) who studied a total of 217 patients with confirmed Argentine haemorrhagic fever. Previous work had shown that the mortality in guinea pigs infected with Junin virus may be considerably reduced by passive transfer of antibodies (Carballal and Frigerio, 1973).

All patients studied by Maiztegui and colleagues entered the trial within 8 days after the onset of illness and in 188 patients the diagnosis was confirmed by laboratory methods. Immune plasma was drawn from donors convalescent from Argentine haemorrhagic fever in whom at least a 4-fold rise in antibody titre had been demonstrated previously. The results obtained by Maiztegui et al. clearly show a marked improvement in the mortality rate in patients who received immune plasma as compared to the control group (Table 10.1). Apart from the clear beneficial effect of the use of immune plasma, two important points emerged from this study. Firstly, this improvement was seen in patients treated early in the course of illness: retrospective analysis of a further 24 patients who received immune plasma on day 9 or later showed the same mortality rate as patients receiving normal plasma. Secondly, a number of patients successfully treated with immune plasma were readmitted 2 to 3 weeks later

TABLE 10.1.
Treatment of Argentinian haemorrhagic fever with immune plasma

	Total	Improved	Died
Immune plasma	91	90	1
Normal plasma	97	81	16

Data from Maiztegui et al. (1979).

with a late neurological syndrome characterized by cerebellar manifestations and fever of short duration. The majority of these relapses were benign and self-limiting, although a severe to fatal neuropathy was seen. Maiztegui and colleagues have pointed out the parallel with the studies of Eddy et al. (1975b) in monkeys experimentally infected with Machupo virus after passive immunization. As discussed above, the protected monkeys did not develop haemorrhagic illness on challenge with the virus, although many animals exhibited a late neurological syndrome some weeks later. It is possible that this complication is directly related to immunoglobulin dose, at least in the case of Machupo-infected primates (Eddy and Cole, 1978).

In a retrospective study of Junin patients treated with immune plasma, Enria and colleagues (1984) have determined that the neutralizing antibody titre in individual lots of administered plasma is a critical factor. Only 3 of 30 survivors so treated had received plasma with a low antibody titre. However, 4 of 7 patients who died after infusion of immune plasma had received a comparatively low dose of neutralizing antibody. The major effect of plasma administration is a marked reduction in viraemia, although neutralizing antibody may also play a role in controlling host lymphokine secretion during acute illness. For example, there is a marked decrease in the amount of circulating alpha-interferon in patients receiving immune plasma, presumably as a result of either restricting further virus spread to macrophages or leucocytes or by direct lysis of infected cells after recognition of viral antigens at the cell membrane (Levis et al., 1984).

The indications for specific immunotherapy of Lassa fever patients has been discussed by Monath et al. (1974b). Patients with persistent high fever and signs of capillary leakage are regarded as having a particularly poor prognosis and would be prime candidates for treatment. Although such selection is arbitrary, the limited availability of plasma is a major factor in determining the use of immunotherapy.

Additional factors in the development of Lassa fever immunotherapy

include an objective assessment of the degree of protection versus titre of specific antibody and the extent of any antigenic variation in Lassa virus isolates. The occurrence of Lassa virus subtypes would raise the possibility of limited protection if antibody was administered from a person infected with a heterologous subtype. A further consideration is the screening of immune plasma for markers of viral hepatitis agents. For example, the endemic area of Lassa fever closely corresponds with a high carriage rate of hepatitis B virus which in some countries in West Africa frequently exceeds 10% of all individuals (Szmuness, 1978).

Some of these problems concerning immunotherapy have recently been addressed by Jahrling (1983), who examined the protective efficacy of immune plasma obtained either from convalescent patients or animals experimentally infected with Lassa virus. Guinea pigs, normally susceptible to infection, were passively protected with plasma regardless of origin, providing the neutralizing antibody titre in each case exceeded 2 logs as measured *in vitro* by a plaque-reduction test. This is similar to the findings of Maiztegui and colleagues who found that immune plasma treatment of patients with Argentine haemorrhagic fever was most effective using lots with a high titre of neutralizing antibody (Maiztegui et al., 1979; Enria et al., 1984). As with Junin virus, it is important to note that Jahrling found a positive correlation between protection and neutralizing antibody titre but this relationship was not maintained by substitution of immunofluorescence as the method for antibody titration. Immune plasma obtained on or before the 45th day from a convalescent human case failed to protect susceptible guinea pigs, even though the titre of this preparation by immunofluorescence exceeded 1:2560. In contrast, plasma taken at 90 days conferred protection, although the immunofluorescence titre remained unchanged. In these animals there was no sign of viraemia. Progressive dilution of immune plasma resulted in less than 100% protection and a corresponding rise in viraemic levels resulting from the reduction in the amount of specific antibody administered.

Efficacy testing in infected cynomolgus monkeys has shown a similar correlation between the titre of neutralizing antibody, volume of immune plasma administered and protection (Jahrling and Peters, 1984). This study clearly confirmed that neutralizing antibody as measured by *in vitro* methods and protective efficacy was rapidly lost on dilution. Monkey immune plasma diluted to a level of neutralizing antibody typical of human convalescent plasma failed to protect when administered on three to five separate occasions during the development of the disease. Additionally, the total quantity of plasma used rather than the volume appeared to be an important variable; immune plasma with only margi-

nal significant titres against the virus may still prove useful if infused in a sufficiently large volume early in infection. Plasma diluted below the level known to confer protection did not have a significant effect on viraemia, suggesting that low levels of passively administered antibody does not result in an enhancement effect mediated by a low immunoglobulin concentration as might be expected from studies with flaviviruses. Furthermore, repeated use of plasma at low titre did not achieve the same level of protection as with the use of an equivalent single dose administered early after exposure to the virus.

In contrast to the experience with the treatment of Machupo-infected rhesus monkeys with immunoglobulin, there appeared to be no signs of any late neurological involvement in those animals recovering from acute infection as a result of having received Lassa immune plasma. Monkey immune plasma diluted to a level of neutralizing antibody typical of human convalescent plasma failed to protect when administered on three to five separate occasions during the development of the disease.

Some slight difference was seen in the degree of protection using plasma containing antibodies to different strains of Lassa virus. One plasma sample from a patient infected in Liberia was found to neutralize over 1 log more virus of a Liberian isolate as compared to a reference strain obtained originally from neighbouring Sierra Leone. This same sample effectively protected both guinea pigs and cynomolgus monkeys infected with either strain, although quantitatively less was required to protect animals challenged with the Liberian isolate (Jahrling and Peters, 1984).

The combined use of immune plasma and ribavirin may enhance the degree of success in the treatment of Lassa fever. All infected monkeys given this combination by Jahrling and colleagues (1984) survived when treated for the first time either on the day of infection or up to 10 days later. Animals infected on the first day never developed a viraemia although an antibody response 6 weeks later suggested the development of a subclinical infection. Those treated later subsequently developed a viraemia, albeit at a much lower level compared to untreated, infected monkeys. In each instance, combined antibody-antiviral therapy was more effective than either ribavirin or plasma alone. Although the reasons for this synergism is unclear, passive immunization only appears to suppress viraemia effectively without significantly reducing the level of virus replication in target tissues whereas ribavirin concentrates in tissues identified as major sites of virus replication with the result that significantly less virus was recovered from the spleen, liver, lung and other organs (Jahrling and Peters, 1984). Combined therapy for human cases

of Lassa is particularly attractive for two reasons. Firstly, much of the immune plasma available is of low titre and its effect could be usefully enhanced by simultaneous antiviral treatment. Secondly, early intervention is desirable but often not possible. The initiation of combined therapy as late as 10 days after infection in monkeys appears to have a significant effect on viraemia. Taken together, these results show that passive prophylaxis and immunotherapy may be used to control human arenavirus infections. Studies have clearly shown that acutely ill patients undergo a marked improvement in condition, provided immunoglobulin is given early during the infection and has been clearly shown to contain viral antibodies. The use of the neutralization test is critical in this assessment, it being insufficient to rely on serological tests such as immunofluorescence or the less sensitive CF procedures. Good results have been obtained by collecting immune plasma at least 3 months after illness when neutralizing antibody levels have increased and the risk of trace amounts of infectious virus can be discounted. Early convalescent sera from either humans, primates or guinea pigs frequently contain infectious virus in addition to non-neutralizing specific antibody (Jahrling et al., 1980, 1982; Wulff and Lange, 1975). In the absence of a suitable vaccine it may be anticipated that specific immunoglobulin could be prepared in the foreseeable future for the passive immunoprophylaxis of individuals at high risk, providing suitable care is taken in the selection of donors. It is clear that, despite the clinical benefits of administering immunoglobulin to acutely ill patients, prolonged shedding of virus poses a public health hazard if such patients are discharged prematurely from isolation.

10.3. Active immunization: the development of a vaccine against Argentine haemorrhagic fever

Complete eradication of Junin virus from the endemic area of Argentine haemorrhagic fever will only be achieved once the mechanisms governing persistence of the virus in its natural rodent hosts become known and suitable techniques are applied to eliminate this natural reservoir. For the foreseeable future, efforts are likely to continue towards the development of a suitable human vaccine against the disease in order to protect those living within the endemic area. It is estimated that over 90% of individuals residing in this zone are susceptible to the disease; in numerical terms this was estimated at over 1 million people for the year 1983 (M. Weissenbacher, personal communication). As with the potential control of

Lassa fever, the non-human reservoir of the disease creates a particular difficulty in establishing an efficient level of immunity in the human population; other diseases of man may be controlled by establishing immunity in a high percentage of individuals thus reducing the opportunity of man-to-man transmission. In this instance, however, control of Argentine haemorrhagic fever will only prove effective if almost 100% of susceptible individuals are protected as the level of herd immunity will not decrease the likelihood of any one individual coming into contact with the virus whilst the rodent reservoir remains unrestricted.

Three approaches have been explored toward the development of a Junin virus vaccine. The first of these is the use of inactivated whole virus. Several different methods of inactivation have been proposed, including ultraviolet light (D'Aiutolo et al., 1979), fluorescence in the presence of neutral red (Parodi et al., 1965), or chemical cross-linkage using either methylene blue or 2% formaldehyde (Martinez Segovia et al., 1979; Barrera Oro et al., 1967). The potency and efficacy of each of these preparations has only been tested in the guinea pig model, however, and to date the results have not been sufficiently encouraging to extend the use of inactivated preparations to man. However, an experimental inactivated

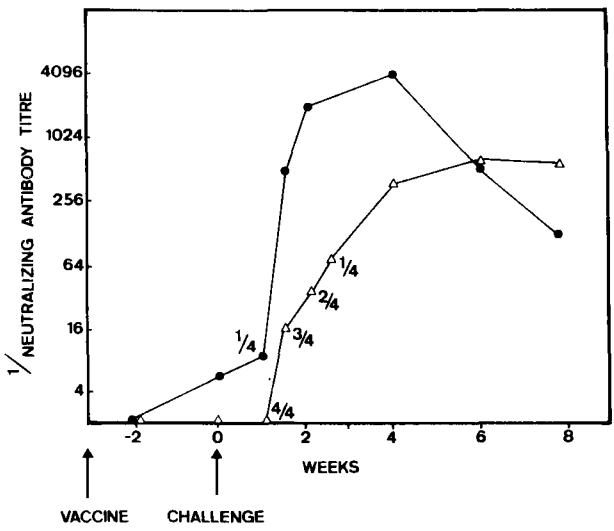

Fig. 10.1. Antibody responses in rhesus monkeys immunized with inactivated Machupo virus 3 weeks prior to challenge with 1000 pfu of live virus. Two groups of animals received either 3.0 ml of the vaccine (•) or 10-fold less (△). Numbers by the plots of geometric mean antibody titres indicate the number viraemic/total number of animals inoculated (redrawn from Eddy and Cole, 1978).

vaccine against Bolivian haemorrhagic fever has been shown to be safe and efficacious using formalin-treated Machupo virus (Eddy and Cole, 1978; Fig. 10.1).

An alternative approach is the use of a split vaccine consisting of viral glycoprotein bearing the antigenic determinants important for stimulating immunity. One such attempt has been made to separate the envelope glycoproteins by non-ionic detergent solubilization of whole virus followed by purification (Cresta et al., 1980). Rabbits inoculated with the solubilized fraction developed neutralizing antibody to the virus and guinea pigs immunized with this fraction were subsequently protected against challenge with whole virus.

Most progress has been achieved in the attenuation of a human isolate of Junin virus isolated from a patient in 1958 and termed the XJ strain (Parodi et al., 1958). This was received by Dr. J.Casals in New York one year later having been passaged twice in guinea pigs and 11 times in suckling mice. After a further 40-mice passages in Dr. Casals' laboratory the XJ virus lost its pathogenicity for guinea pigs (Mettler et al., 1963; see Chapter 5), a virulence marker for this virus (Guerrero et al., 1969).

The XJ-Cl_3 strain of Junin virus is relatively non-pathogenic for guinea pigs, with a mortality of 15–20% compared to the 100% mortality in animals infected with the parent XJ strain (Guerrero et al., 1969; Avila et al., 1981b). Those animals which succumb to the attenuated strain die 3 weeks after infection, showing a much more prolonged survival time. The XJ-Cl_3 virus is found in the spleen and lymph nodes 8–15 days after inoculation, although viral antigen can be found by immunofluorescence in the pancreas, lungs and kidneys of these animals. One prominent feature found by these workers was the presence of virus and lesions in the central nervous system, a finding absent in those animals receiving the XJ strain. Viral antibodies are readily demonstrated in those animals surviving XJ-Cl_3 infection with no detectable viraemia. Protection is also conferred on guinea pigs inoculated by the intranasal route with XJ-Cl_3 virus, producing the same low mortality and degree of virus spread as the intramuscular route (Samoilovich et al., 1983).

Both CF and neutralizing antibodies developed from the third week, although CF antibodies began to wane after 2 months. Noticeable was the appearance of lesions in the central nervous system some 2 weeks after the appearance of circulating antibodies. Whether this has any parallel with the late neurological syndrome in human cases after administration of immune plasma is not clear. The mounting of a specific immune response, not seen in animals infected with the lethal XJ strain, correlates with the lack of immunosuppression: Blejer et al. (1981) found

that animals inoculated with the XJ-Cl_3 virus showed both normal B-cell and delayed hypersensitivity reactions to unrelated antigens. All animals receiving a prior inoculation of the attenuated strain were fully resistant to a lethal dose of XJ virus as early as 10 days after the first infection, ahead of a demonstrable immune response (Guerrero et al., 1969). Susceptible marmoset monkeys are also protected by immunization with the XJ-Cl_3 strain. Although a viraemia was demonstrated in the 2nd and 3rd weeks, there were no signs of illness and the animals were solidly protected on challenge with XJ virus 2 months later (Avila et al., 1985).

An alternative marker of attenuation is the sensitivity of 2-day-old Wistar rats to Junin virus. Whereas intracerebral inoculation with XJ virus results in a subclinical infection without mortality, newborn animals similarly inoculated with the attenuated virus die within 3 weeks with severe encephalitis (Avila et al., 1981b). This response appears to be immunologically mediated as the use of cyclophosphamide and anti-theta serum considerably improves the 5% survival rate in these animals. Further evidence is the protection conferred by administering immune serum to newborn rats 2 h prior to infection. Although this did not directly affect the level of virus replication in the brain, the increased survival rate suggests that the attenuated virus fails to induce an appropriate antibody response essential for recovery in Wistar rats (Blejer et al., 1984).

A variant of XJ virus, termed XJ_0, has also received some attention as a possible candidate vaccine strain by virtue of having a better defined passage history without passage in heteroploid cells. This strain is avirulent for guinea pigs to the same extent as XJ-Cl_3 virus (Boxaca et al., 1980; Guerrero and Boxaca, 1980). One interesting feature from studies with this strain was the further reduction in mortality to 11% in animals infected by the intramuscular rather than the intracerebral or intraperitoneal routes (Boxaca et al., 1980). As with animals immunized with the XJ-Cl_3 strain, guinea pigs immunized with XJ_0 virus are fully protected on challenge with virulent virus (Boxaca et al., 1981). Some progress has been made in adapting the XJ_0 strain to MRC-5 cells, a human diploid cell line widely accepted as a suitable substrate for vaccine production (Weber, E.L. et al., 1983).

Between 1968 and 1970, a total of 636 volunteers were inoculated with the attenuated XJ-Cl_3 virus. In all of these individuals the vaccine induced either a mild febrile reaction or a subclinical infection. The initial study was conducted on seven adults who received doses varying from 5000 to 100 000 infectious units (Rugiero et al., 1969). The individual

inoculated with the highest dose responded with petechiae, adenopathy, fever and headache 6–10 days after inoculation but later recovered completely. Other symptoms were seen in all but two of the remaining individuals and all vaccinees showed a temporary leucopenia and thrombocytopenia although not nearly as severe as seen in clinical cases of Argentine haemorrhagic fever. Neutralizing antibody first appeared at 25 days after immunization and rose steadily in titre over the first 2 months of follow-up. Complement fixing antibody appeared much later and seemed to be of limited duration. Expansion of this trial to individuals in the endemic area showed that over 90% of vaccinated individuals developed neutralizing antibody to the virus within 3 months (summarized in Guerrero, 1977).

A detailed study of some of these volunteers again showed the development of subclinical infection with approximately 44% having minor symptoms about one week after inoculation, which consisted of asthemia, myalgia, headache and retro-orbital pain (Parodi et al., 1970b). A further 21% showed a mild febrile reaction but as with the first group there was no evidence of clinical disease. The duration of the response has been re-examined recently by Rugiero et al. (1981) who found the level of protection was maintained; 90.3% of the vaccinees still had detectable neutralizing antibody 7–9 years after immunization with a single dose of the XJ-Cl_3 virus. Although this may be due to further natural contact with the virus, this appears unlikely as the infection rate in the endemic area is approximately 12% (Weissenbacher et al., 1983). A long-lasting response was also seen in laboratory workers at high risk of infection who had been vaccinated; as with persons among the same group with evidence of past inapparent infections there was no clinical evidence of re-

TABLE 10.2.

Cross-protection studies in guinea pigs against normally lethal Junin virus infection

1st inoculum	2nd inoculum	Average day of death
None	Junin (10^3)[a]	11.8
Tamiami (10^6)	Junin (10^3)[a]	14.5
Amapari (10^5)	Junin (10^3)	16.8
Pichinde (10^6)	Junin (10^3)	17.8
Tacaribe (10^6)	Junin (10^3)	protection
Machupo (10^5)	Junin (10^3)	protection

[a] LD_{50} 2nd inoculum given 30 days after the first.
Data from Weissenbacher et al. (1975/76).

infection, thus emphasizing the efficacy of active immunization against Junin virus.

Detailed analysis of the neutralizing antibody response in animals protected by heterologous vaccination has shown that neutralizing antibodies to Tacaribe virus develop by day 10 and that challenge on day 30 with Junin virus stimulates the production of antibody specific for Junin with all the characteristics of a secondary antibody response (Coto et al., 1980). Continual stimulation of the immune system with Tacaribe virus only resulted in a high antibody titre against the homologous virus which exceeded a neutralization index of over 6 logs; during this time heterologous reactions against Junin virus developed steadily although there was marked variation in the neutralization index between individual animals (Weissenbacher et al., 1975/1976). The highest responses against Junin virus never exceeded or approached that measured with homologous virus, however.

Vaccination against Junin virus may be possible using Tacaribe virus, originally isolated over 3000 miles away from the endemic region of Argentine haemorrhagic fever (Calisher et al., 1970). Both mouse immune and human convalescent sera to Junin virus cross-react with Tacaribe virus by the CF test (Mettler et al., 1963). However, there is some evidence that Junin virus is weakly neutralized by Tacaribe immune serum *in vitro* (Henderson and Downs, 1965; Weissenbacher et al., 1975/1976), and considerable evidence to show that prior immunization of guinea pigs with Tacaribe virus protected against challenge with a virulent strain of Junin (Coto et al., 1976; Weissenbacher et al., 1975/1976; Tauroso and Shelokov, 1965). Animals injected intramuscularly 30 days prior to challenge were solidly protected: various degrees of protection were also observed using other non-pathogenic members of the Tacaribe complex, the most effective being Amapari virus which induced protection in nearly 60% of guinea pigs although three doses of immunogen were required in contrast to the one dose of Tacaribe virus (Coto et al., 1976; Weissenbacher et al., 1975/1976). Although other arenaviruses did not give effective protection, notable was the prolongation of survival times on challenge with Junin virus. For example, animals receiving prior immunization with Tamiami or Pichinde viruses survived 16 and 18 days, respectively as compared to non-immunized animals which died 12 days after exposure to Junin virus (Table 10.2).

The protective effect of Tacaribe virus in guinea pigs may be demonstrated as early as 3 days after immunization before the appearance of cross-reactive neutralizing antibodies in the sera (Damonte et al., 1978a). Protection against virus challenge on day 3 correlates with a marked

reduction in the extent of Junin virus replication in all major sites of virus replication. However, protection was abolished by the simultaneous inoculation of both Tacaribe and Junin, suggesting that viral interference does not play a role in the cross-protection. Neutralizing antibodies to Tacaribe virus in immunized guinea pigs develop from day 5. Unexpectedly, however, protection is temporarily lost between 12 and 14 days after Tacaribe virus infection, presumably as a result of interferon production and the stimulation of non-specific immune effector cells (Coto et al., 1980). The protective effect as manifested by challenge after this time appears to be the result of priming the immune system by Tacaribe viral antigens bearing a close resemblance to the relevant determinants on Junin viral proteins; control animals inoculated with Tacaribe virus only develop cross-reactive neutralizing antibodies to Junin virus 2 months later but challenge with Junin virus stimulates the appearance of Junin antibodies in a manner typical of a secondary response (Damonte et al., 1978a). This anamnestic response is seen on challenge also with inactivated Junin virus and confirms the essentially antigenic nature of this response and that Junin virus replication is not required. Indeed, as yet there is no evidence of Junin virus replication although limited replication of Tacaribe virus in the lungs, lymph nodes and spleens of immunized guinea pigs has been reported (Weissenbacher et al., 1977).

Weissenbacher and colleagues (Weissenbacher et al., 1982; Samoilovich et al., 1984) have reported that 10^5 $TCID_{50}$ of Tacaribe virus fully protects the marmoset monkey *Callithrix jacchus*, known to be highly susceptible to Junin virus (see Chapter 5). Careful study of animals prior to challenge failed to reveal any pathology associated with the use of Tacaribe virus as an immunogen and no viraemia was detected. The serological reactions in marmosets differed somewhat from the findings obtained using guinea pigs. Antibodies against Junin virus were not detected prior to challenge: after challenge there was a brisk response to Junin although the titres of neutralizing antibody against Tacaribe virus remained relatively constant. Interestingly, the appearance of neutralizing antibody in the serum of immunized animals does not necessarily confer full protection.

It remains to be determined if the use of Tacaribe virus would indeed be a suitable alternative live vaccine for Argentine haemorrhagic fever, although one problem might be its adaptation to a suitable human diploid cell substrate: growth of Tacaribe virus in MRC-5 cells rarely exceeds 2×10^4 pfu/ml although such virus still possesses the capacity to immunize guinea pigs against Junin (Damonte et al., 1981a). Although the possible reversion that may occur with attenuated Junin virus vaccines would be

eliminated on the basis that Tacaribe virus is thought to be non-pathogenic for man, at least one laboratory-acquired infection has been suspected and reported by the Sub-Committee on Arbovirus Laboratory Safety (1980). Therefore considerable caution is required in the development of Tacaribe for human use and extensive evaluation in primates would be valuable. Also, information acquired concerning Tacaribe-Junin cross-protection would be directly relevant in considerations of Mopeia as a candidate vaccine for Lassa virus. In either instance, however, it may be appropriate now in the light of recent advances in vaccine technology to reconsider the use of a killed vaccine, against Argentine haemorrhagic fever.

10.4. Prospects for the development of a Lassa fever vaccine

Attempts to produce vaccines against the Old World arenavirus have not progressed beyond efficacy studies in experimental animals, although efforts to produce an inactivated vaccine against LCM virus were in progress as early as the 1940s. For example, Traub (1937) had noticed that various LCM virus isolates from infected laboratory mice differed in virulence for guinea pigs. Some caused a mild febrile illness, whereas others induced a severe fatal disease. One such strain found to be pathogenic for guinea pigs was attenuated in virulence by the eighth serial passage in mouse brains; none of the 53 animals died as a result of inoculation with the passaged virus in contrast to the 80% or more mortality with the parental virus. Moreover, virulence was not restored after a further passage in guinea pigs. Early attempts to develop an inactivated LCM virus vaccine were only partially successful (Traub, 1938; Jocheim, 1957), perhaps because the technology for assessing the kinetics of virus inactivation by chemical agents had not been sufficiently developed. A more successful approach has been the selection of a LCM variant with reduced virulence for animals. The variant was obtained from mouse L cell cultures persistently infected with the WE strain (Hotchin and Sikora, 1973). Modest titres of CF antibody were induced in guinea pigs and almost all animals so protected were resistant to challenge with WE virus even before neutralizing antibody became detectable. Route of inoculation seemed unimportant, although highest antibody responses were obtained using infected cells as the basis of the immunogen together with a multiple injection regime (Hotchin et al., 1977). This slow growing variant produced large amounts of viral antigen in the cytoplasm of infected cells, although there was little evidence of antigen at the plasma

membrane. A correspondingly low level of virus was removed from immunized animals, with no evidence of reversion to wild-type virus. One problem with this variant, however, was the loss of ability to produce plaques in infected cell cultures. Quantitation was best achieved by the somewhat cumbersome method of mouse-inoculation followed by endotoxin challenge (see Chapter 3).

Kiley and colleagues (1979) have reported that closely related arenaviruses non-pathogenic for primates may induce protection against Lassa virus in much the same way as the use of Tacaribe virus is known to protect susceptible animals against normally lethal Junin infection. The Mopeia virus from Mozambique was found to produce mild or no fever in rhesus monkeys with no clinical signs of disease. In contrast, animals exposed to Lassa virus died within 3 weeks after the development of a haemorrhagic fever (see Chapter 5, page 71). However, animals inoculated with the Mopeia virus 75 days prior to infection with Lassa virus rose up to 8-fold within 2 weeks, suggestive of an anamnestic response. Pathological studies have shown that the extent of tissue injury was much reduced in the animals inoculated with Mopeia virus prior to challenge, although the degree of inflammation was enhanced as compared to that seen in animals with a primary Lassa virus infection (Walker et al., 1982). This took the form of focal mononuclear cell infiltrates in areas of hepatic necrosis with additional infiltrates in the renal interstitia. More disturbing was the finding of scattered focal lesions in the brain of Mopeia-protected animals pointing out that the use of a live arenavirus vaccine may itself pose inherent problems as to establishing with certainty its non-pathogenic nature. A further consideration is the theoretical possibility that such a vaccine may establish a low level persistent infection, even in the presence of circulating antibody. The continuing high levels of neutralizing antibody seen in individuals inoculated with an attenuated Junin virus vaccine illustrate that this may be a real possibility although this may be acceptable in individuals at high risk of acquiring the infection if persistence is not accompanied by pathological signs or illness.

Protection using closely related arenaviruses implies a close antigenic relationship between pathogen and the candidate vaccine virus. All evidence indicates that Mopeia virus represents a variant of Lassa virus with reduced virulence for man. Notably, more distantly related viruses offer no protection in non-human primates. For example, animals receiving Flexal virus from Brazil were not significantly protected from Lassa infection (Walker et al., 1982). It remains to be seen if Mopeia virus will be shown experimentally to protect equally well animals challenged with

different strains of Lassa virus; subtle differences between Lassa isolates obtained from different geographical areas are now becoming apparent (Jahrling and Peters, 1984) and the extent of variation may be sufficient to abrogate the protective effect of Mopeia vaccination in the case of certain strains. Considerably more effort is thus required before these findings can be extended to clinical trials in man. One further difficulty is that Mopeia virus grows less readily in tissue culture which may present problems in the bulk preparation of a potential vaccine.

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