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Arenaviruses: Genes, Proteins, and Expression

Arenaviruses

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Edited by M. B. A. Oldstone

With 33 Figures

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The Arenaviruses – An Introduction

M.B.A. OLDSTONE

Viruses are generally studied either because they cause significant human, animal or plant disease or for their utility as materials to probe a basic phenomenon in biology, chemistry, genetics or molecular biology. Arenaviruses are unusually interesting in that they occupy both of these categories.

Arenaviruses cause severe human diseases known primarily as the hemorrhagic fevers occurring in South and Latin America (Bolivia: Machupo virus and Argentina: Junin virus) and in Africa (Lassa virus). Because such viruses produce profound disability and may kill the persons they infect, they are a source of economic hardship in the countries where they are prevalent. Further, they provide new problems for health care personnel owing to the narrowing of the world as visitors from many countries increasingly travel to and from these endemic areas. In addition, lymphocytic choriomeningitis virus (LCMV) can infect humans worldwide, although the illness is most often less disabling than those elicited by other arenaviruses. Yet LCMV is likely of greater concern to non-arena-virologists and experimentalists using tissue culture or animals, i.e., workers in molecular biology, cancer research, virology, immunobiology, etc., because normal appearing cultured cells or tissues and animals used for research may be persistently infected with LCMV without manifesting clinical disease or cytopathology and transmit that infection to laboratory workers (reviewed OLDSTONE and PETERS 1978). For example, HINMAN et al. (1975) recorded 48 cases among personnel in the radiotherapy department and vivarium at the University of Rochester School of Medicine. These persons had contact with Syrian hamsters into which tumors, unknown to be occultly infected with LCMV, were transferred. These tumors were obtained from an outside research supplier who distributed tumor cell lines to numerous laboratories primarily interested in SV-40 and polyoma virus research. In subsequent investigation of the 22 tumor lines in question, 13 yielded infectious LCMV. Infection was spread from the experimental area, presumably via air ducts. Similarly, the Communicable Disease Center, USA, has recorded multiple cases of LCMV infections in families scattered from the northeast coast of the United States (New York) to the western region (Reno, Nevada) originating from hamsters sold by a single supplier in the southeast (Florida). Several investigators found that their hybridomas making monoclonal antibodies were infected with LCMV. The mechanism was likely by the use of infected splenic feeder cells obtained

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Table 1. Recognized Arenavirus diseases of man

Virus	Disease	Locality	Rodent reservoir and vector	Person to person transmission	Laboratory model of human infection
Lymphocytic choriomeningitis	Grippe, aseptic meningitis, occasional more severe forms of meningoencephalomyelitis	Probably originated in Europe, now worldwide	Mus musculus natural host; colonized rodents, particularly mice and hamster; dog?	Never documented	Mouse
Junin	Argentinian hemorrhagic fever	Circumscribed area of Argentina; Buenos Aires to northeast	Calomys musculinus, possibly others	Occasional	Guinea pig
Machupo	Bolivian hemorrhagic fever	Beni region of Bolivia	Calomys callosus	Occasional, particularly spouses; recognized hospital outbreak	Rhesus monkey
Lassa	Lassa fever	Western Africa	Mastomys nataliensis	Frequent; blood contamination	Squirrel monkey, Guinea pig strain 13 LCMV WE in guinea pig

from clinically healthy but persistently infected mice purchased from a commercial source. Hence, researchers studying such diverse areas as the molecular biology of SV40, biologic effects of chlamydia, immune responses of mice making hybridoma cells or production of ascites fluids have found their preparations contaminated with LCMV (Lehmann-Grube 1973; WHO Bulletin 1975; Grimwood 1983, 1985; Van der Zeijst et al. 1983a, b). Thus, experimentalists may be exposed to the unexpected and potentially dangerous effects of LCMV owing to its non-cytolytic persistent infection in tissue culture cell lines and experimental or pet animals. The recognized arenavirus diseases of man, the vectors and laboratory models used for their study are listed in Table 1.

Study of the biology of LCMV has led to several major advances in contemporary virology and immunology. For example, research on both the acute and persistent infection of mice with LCMV led to the first descriptions of virus induced immunopathologic disease (Rowe 1954), of T cell mediated killing (Cole et al. 1973; Marker and Volkert 1973) and of the two unique and separate signals necessary for recognition and lysis of virus infected targets by cytotoxic T lymphocytes (CTLs) (Zinkernagel and Doherty 1974). Hence, study of the LCMV indicated that CTLs kill virus infected targets only when two conditions are met: recognition of virus specific determinants and matching of the major histocompatibility complex on the CTL and the infected target cell. This phenomenon of MHC restriction, one of the major tenets of contempo-

rary immunology, was not only first described by experimentalists working with LCMV but also its implications were clearly recognized in this setting (reviewed by ZINKERNAGEL and DOHERTY 1979). Additionally, the components, kinetics, genetic control and deposition of immune complexes from the circulation into a variety of tissues including renal glomeruli, brain choroid plexus and blood vessel arteries were explored by utilizing the LCMV model (OLDSTONE and DIXON 1969). The conclusions were extended to work on numerous RNA and DNA virus infections as well as infections with other microbial agents (reviewed OLDSTONE 1975). Recently, by examination of its supposedly benign effect, LCMV was shown to cause disease not by means of the well-known anatomical viral induced destruction of infected cells, but rather by disordering the synthesis of such cells' differentiated products (OLDSTONE et al. 1982; reviewed OLDSTONE 1984). These studies in animals indicate that viruses may cause disease by altering the cells' physiology and function without causing their destruction. If these findings are applicable to human viruses, in vivo, we can anticipate much-needed understanding of the mechanisms by which dysfunctions of endocrine, nervous and immune systems occur.

Arenaviruses obtained their name from arenosus – Latin for sandy – on the basis of characteristic fine granularities seen inside the virion on ultrathin section (ROWE et al. 1970). To avoid confusion between areno and adeno viruses, the International Committee on Viral Nomenclature changed the name to arenaviruses (ROWE et al. 1970).

Recently, the ability of such viruses to cause persistent infection in cultured cells and animals, as well as their non-cytolytic nature, has been coupled with the wealth of information known about their biologic activity to inspire intensive investigation into the molecular control of viral genes and the molecular basis of arenavirus induced disease. It is the melding of newly obtained information on the viral chromosomes and gene structure, the proteins encoded by various viral genes and the ambisense (positive-genomic sense and negative-complementary sense) organization of the arenavirus genome – particularly as relates to the molecular basis of viral persistence, tissue tropism, viral gene regulation and mechanism of pathogenesis and biology of the virus – that led to the selection of arenaviruses for these two volumes in the Current Topics in Microbiology and Immunology series.

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Experimental Studies of Arenaviral Hemorrhagic Fevers*

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* In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals", as promulgated by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council. The facilities are fully accredited by the American Association for Accreditation of Laboratory Animal Care. The views of the authors do not purport to reflect the positions of the Department of the Army or the Department of Defense. Approved for public release; distribution unlimited.

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1 Introduction

Three arenaviruses, Lassa, Junin, and Machupo, regularly cause serious disease when they infect humans: Lassa fever, Argentine hemorrhagic fever (AHF), and Bolivian hemorrhagic fever (BHF), respectively. There is increasing acceptance that these and other human viral infections share sufficient, common, clinical and pathophysiological findings to refer to a viral hemorrhagic (HF) syndrome (JOHNSON and PETERS 1985). This entity is characterized by fever, malaise, prostration, and evidence of diffuse vascular dysregulation and permeability changes. Hemorrhagic manifestations occur in many cases, usually in proportion to the gravity of the illness and the prognosis. The mechanisms underlying these clinical events probably vary depending on the infecting virus, but almost certainly share common themes (PETERS and JOHNSON 1984). The biohazard of the causative viruses, the difficult logistics of clinical research in the regions where most HFs occur, and the limited international support for research on so-called "exotic" viruses have all been important barriers to progress in the field.

This chapter discusses animal and cell culture models of arenaviral infection which contribute to our understanding of the pathogenesis, immunology, and epidemiology of the human diseases. It is important to recognize that neither the classical T-cell mediated encephalitis seen in immunocompetent mice inoculated i.c. with lymphocytic choriomeningitis virus (LCMV) nor the chronic immune complex disease seen in LCMV-carrier mice necessarily share pathogenetic similarities with HF. Indeed, a major stumbling block to the laboratory analysis of arenaviral HF is the lack of a realistic mouse model and the consequent

reliance on less thoroughly characterized and more expensive species, such as guinea pigs and nonhuman primates.

2 Animal Models

2.1 Lymphocytic Choriomeningitis Virus

2.1.1 Mouse

Human LCMV infection results in a spectrum of clinical outcomes, one of which is acute aseptic meningitis (PETERS 1984). This syndrome has several parallels with the outcome of i.c. inoculation of adult mice: clinical manifestations coincide with the onset of the immune response and in both situations immunosuppressed hosts are spared neurological disease (HORTON et al. 1971).

Of more relevance to the human HF syndrome, however, are the physiological alterations that occur following i.p. inoculation of mice with certain LCMV strains (ROWE 1954). This same syndrome of tachypnea, pleural effusions, and other evidence of increased vascular permeability has also been observed in cyclophosphamide (CY)-treated mice infected with a strain of LCMV not pathogenic for intact animals (JOHNSON et al. 1978). Exploration of the pathogenesis of these lesions might provide a useful murine model for VHF.

2.1.2 Hamster

LCMV has been shown to cause acutely lethal infection, subacute disease, and asymptomatic infection of outbred hamsters (PARKER et al. 1976). Systematic studies of WE and mouse-adapted Armstrong (ARM) strains of LCMV have shown major virus-host genetic interactions (GENOVESI, JOHNSON, and PETERS, unpublished observations). Several plaque-cloned isolates tested in outbred hamsters resembled the parental stock. Typical virulent WE and avirulent ARM plaque-cloned viruses were inoculated i.p. into inbred hamsters. The ARM clone was uniformly avirulent. The WE clone resulted in death of MHA and PD4 hamsters within 10–20 days after inoculation, while CB and LSH hamster strains suffered no overt disease, survived indefinitely, and cleared infectious virus from their serum and organs. About half of inbred LHC and outbred LVG hamsters succumbed to the same clinical syndrome as susceptible inbred strains, and about half suffered no discernible illness. Susceptible animals had higher viral titers in serum and internal organs than did resistant animals (6–7 $\log_{10}$ vs 3–4 $\log_{10}$ plaque-forming units (PFU)/ml or g, respectively).

Viremia and organ virus burdens began to decline in the 2nd or 3rd week of infection, particularly in animals destined to survive. Indirect flourescent antibodies (IFA) were detectable by the 1st week of infection regardless of outcome. By the 4th week, viremia was no longer detectable in surviving animals and low levels of neutralizing (N) antibody were present. The neutralization

reaction resembled that seen in Lassa fever (Sect. 5.2). The inoculation of WE in the foot pad of hamsters led to the same strain-dependent mortality patterns that followed i.p. infection. Hamsters of resistant strains developed prominent foot pat swelling around day 7 with a histopathological pattern of hypersensitivity; susceptible animals had no response and died. Infection by foot pad inoculation of ARM followed the same benign course as by i.p. injection, but no local evidence of DTH was observed.

Immunosuppression of WE- or ARM-infected hamsters with CY or gamma irradiation suppressed IFA, N, and DTH responses. All WE-infected, immunosuppressed hamsters died, regardless of strain type. Thus, an effective immune response was necessary for recovery from WE, and lethal disease could occur in the absence of detectable antibody or DTH responses. Although effectively immunosuppressed, some ARM-infected hamsters of each genotype survived.

LCMV infected every organ examined, but particularly the spleen, liver, lung, kidney, adrenal, uterus, and brain. Viral antigen was found predominantly in vascular or perivascular sites, usually in the distribution of tissue or sinusoidal macrophages. There was little or no necrosis, and inflammation was confined to patchy, perivascular, mononuclear cell infiltrates; spleen and lymph nodes were enlarged due to lymphoid hyperplasia. Moribund, WE-infected MHA and PD4 hamsters exhibited ileal congestion with proliferative epithelial changes. This correlated with the occurrence of wasting and diarrheal disease, but none of the gross and histological findings of transmissible ileal hyperplasia or "wet-tail" were present. There was no specific histological lesion in any organ which would explain the fatal outcome, but lethal disease was always associated with remarkable wasting. These young adult hamsters typically underwent a 30%–40% weight loss before dying, in contrast to a 30%–50% growth of resistant or control animals.

Thus, LCMV infection of the hamster provides a useful model of arenaviral HF with several similarities to human Lassa fever.

2.1.3 Guinea Pig

LCMV is highly infectious for outbred guinea pigs, but the lethality of commonly used laboratory strains varies from that of WE ($LD_{50} < 1$ PFU) to that of the mouse-adapted ARM strain ($> 6 \log_{10}$ PFU failed to kill). Guinea pigs infected with WE typically begin to lose weight by day 8, and by day 12 hindlimb paralysis is the dominant clinical sign, immediately preceding death. There are high titers of infectious virus ($> 6 \log_{10}$ PFU/g) and antigen accumulation in the spleen, lungs, adrenal, liver, kidney, and brain. Lesions include necrosis of both red and white pulp of the spleen, fatty infiltration of the liver with variable degrees of necrosis, and focal necrosis of the adrenal cortex. Despite high titers of infectious virus in the kidney and brain, minimal evidence of histopathological lesions is observed. Thrombus deposition in the lungs, spleen, and liver is common. Viral antigens are frequently detected in peritubular areas of the kidney, but rarely in the lumens; those present in the glomeruli may represent viral replication in mesangial cells, or immune complex deposition.

Endothelial cells of blood vessels in myocardium and brain parenchyma are also involved (BUCHMEIER, pers. comm.). Massive accumulations of viral antigens have also been observed in transitional epithelium of the bladder, which may be the source of infectious LCMV in the urine of LCMV WE-infected guinea pigs.

In contrast, ARM infections are benign and characterized by minimal to undetectable replication and antigen accumulation in target tissues. LCMV antibodies are detectable by IFAT or ELISA within 7–10 days of inoculation and a cytolytic spleen cell response within 10–15 days, but serum N antibody only appeared several weeks later. Immunosuppressive CY regimens have no effect either in exacerbating ARM infection or in protection against WE.

2.1.4 Monkey

LCMV WE produces a rapidly fatal infection in both rhesus and cynomolgus monkeys after inoculation by peripheral, i.c. , or aerosol routes (DANES et al. 1963). In our laboratory, six cynomolgus monkeys died 13–14 days after s.c. inoculation with 1000 PFU of virus (JAHRLING, unpublished observations). All monkeys became febrile and anorectic within 7 days of infection. Fevers were maintained at 102°–104° F (39°–40° C) until several hours before death, when body temperatures dropped precipitously. Viremias were first detected on day 3, increased to 5 $\log_{10}$ PFU/ml by day 7, and reached a level of 7–8 $\log_{10}$ PFU/ml, where they remained until death. All animals developed IFA within 10 days, and attained titers of 80–160 by the day of death. Virus was excreted in the urine (3 to >6 $\log_{10}$ PFU/ml), beginning on day 6 and continuing until death.

SGOT concentrations increased tenfold to a mean of 350 IU/dl on day 10. Creatine phosphokinase (CPK) concentrations in serum were very high (>1000 IU/dl). A transient leukopenia, predominantly a lymphopenia, reached a nadir of 3300 by day 7, then reversed to a leukocytosis (predominantly neutrophils) by day 13 (10000–30000/mm^3). Fibrin degradation products were detectable in serum only on day 13. From day 10 until death, all monkeys had petechiae and ecchymoses distributed over most of the skin and a blood-tinged exudate from the nares.

At necropsy, all monkeys had dark red mottling of the lungs, pale yellow livers, and markedly congested spleens. The pericardial sacs and thoracic cavities contained large quantities of straw-colored fluid. High concentrations (7–8 $\log_{10}$ PFU) of infectious virus were found in all tissues examined except brain. By direct immunoflourescence staining, massive concentrations of LCMV antigens were detected in kidney tubules (but not in glomeruli), adrenal cortex (but not medulla), pancreatic and salivary acinar cells, lung, and splenic white pulp. Occasional foci were observed in hepatocytes. In brain, viral antigens were observed clustered around blood vessels (presumably vascular endothelium and/or inflammatory cells).

In contrast, six cynomolgus monkeys inoculated with LCMV ARM had an uneventful course. Viremia was not detected; leukocyte counts and serum chemistries remained within limits. IFA were negative on day 10 but positive

by day 13, and increased to titers of 1280–2560 by day 24. As observed for the Lassa virus survivors (see Sect. 2.2.3 below), the neutralizing antibody response was markedly delayed. Neutralizing antibody titers, measured in a plaque reduction test with Vero cells, were not detectable until 35–50 days after inoculation but, like Lassa antibodies, continued to increase for 90 days or even longer.

The remarkable similarity of LCMV WE infection of macaques to human Lassa fever reminds one that LCMV has on occasion caused fatal human HF-like infections (SMADEL et al. 1942).

2.2 Lassa Virus

2.2.1 Mouse

When Lassa fever virus was initially isolated from humans, the resemblance of its behaviour outbred "white" mice to that of LCMV was noted (BUCKLEY and CASALS 1970). Newborn mice developed inapparent infections with high virus titers in the brain, lung, and muscle, while i.c. inoculated adult mice developed a fatal convulsive disorder resembling classical murine LCM. Subsequently, investigators in other laboratories have failed to reproduce these findings. The apparent discrepancy may be resolved by noting that the outcome of infection depends critically on mouse genotype (PETERS 1984; LUKASHOVICH 1985). Although ths CNS disease is not directly relevant to HF research, the need for a model with such a well-defined immune system is considerable. Further exploration of Lassa and its relatives in the mouse could contribute to our knowledge of viral antigenic relations, T-cell participation in the pathogenesis of disease, and vaccine development.

2.2.2 Guinea Pig

Pathogenicity of Lassa virus for guinea pigs depends both on the virus and the host strain. For example, the Josiah strain of Lassa virus has an LD_{50} of 0.3 PFU for strain 13 guinea pigs, but only kills approximately 30% of outbred Hartley animals receiving between 2 and 200000 PFU (JAHRLING et al. 1982). Viral titers are higher in strain 13 guinea pigs than in outbred animals in all target tissues. Humoral IFA responses were essentially the same in magnitude and timing for both groups. As in monkeys and human Lassa fever patients, neutralizing antibody (log neutralization index, LNI) responses evolved very slowly, first becoming detectable long after the viremia had cleared.

In lethally infected strain 13 guinea pigs, leukopenia was not severe, with average lows of 6200; however, there was a profound lymphopenia, offset by a neutrophilia. Hemoconcentration occurred but was modest and transient. Peak viral titers of 8–9 $\log_{10}$ PFU occurred in the spleen and lymph nodes at 8–9 days, in the salivary glands at 11 days, and in the lung at 14–16 days. Virus reached only low titers in the plasma and brain, and intermediate titers in the liver, adrenal glands, kidney, pancreas, and heart. Histologically, the

most consistent lesion was a moderate interstitial pneumonia. Interpretation of histological lesions was complicated by the presence of bacterial colonies in tissues from more than of the moribund animals, perhaps a significant factor in the pathogenesis of the later stages of this disease and an indication of generalized immunosuppression.

The strain 13 guinea pig has proved a valuable inbred animal model for pathogenetic studies and for testing antiviral drugs and immunotherapeutic regimens. Its high susceptibility to Lassa virus and related viruses provides an alternative to macaque testing for virulence. Several Lassa isolates from sick humans, however, are guinea pig benign, and two of these were lethal when tested in cynomolgus monkeys (JAHRLING et al. 1985a), showing a need for continued primate testing.

2.2.3 Monkey

A spectrum of disease is observed when several species of nunhuman primates are infected with Lassa virus (JAHRLING, unpublished observations). Rhesus, cynomolgus, and African green monkeys usually succumb to inoculation with 6 $\log_{10}$ PFU of the Josiah strain, while squirrel and capuchin monkeys sustain only mild infections; viremia patterns reflect differences in mortality (Table 1). When susceptibility to lower doses was tested, rhesus monkeys inoculated with 1.1 $\log_{10}$ PFU were uniformly killed (6/6), while in contrast only 6 of 10 inoculated with the high dose died. To determine if other primate species showed such an "interference" phenomenon with high doses of Lassa virus, African green and capuchin monkeys were also inoculated with 1.1 $\log_{10}$ PFU. African greens were uniformly killed by the lower dose (as with the higher doses), while capuchins were still uniformly resistant. Monkeys of all species, including those that died, seroconverted (IFAT) even while they were still viremic, suggesting

Table 1. Viremia and lethality in primates inoculated with Lassa virus (Josiah strain given s.c.). <, <0.7 (undetectable)

Species	Inoculum ($\log_{10}$ PFU)	Dead/ total	Geometric mean viremia ($\log_{10}$ PFU/ml) by days						
			3-4	5-6	7	9-10	12-13	16-17	21-24
Rhesus	6.1	6/10	0.9	2.8	3.7	4.2	4.4	3.0	1.7
	1.1	6/6	1.3	–	3.0	5.3	6.3	6.5	[a]
Cynomolgus	6.1	12/12	2.2	4.1	–	5.2	6.1	4.2	2.6
African green	6.1	2/3	2.8	–	4.6	5.6	5.5	3.6	2.9
	1.1	3/3	2.2	–	3.6	4.8	4.6	4.2	[a]
Capuchin	6.1	0/3	<	–	2.6	2.6	2.1	1.8	<
	1.1	0/4	<	–	<	<	<	<	<
Squirrel	6.1	0/4	<	1.8	–	1.9	1.2	1.2	<

[a] All dead

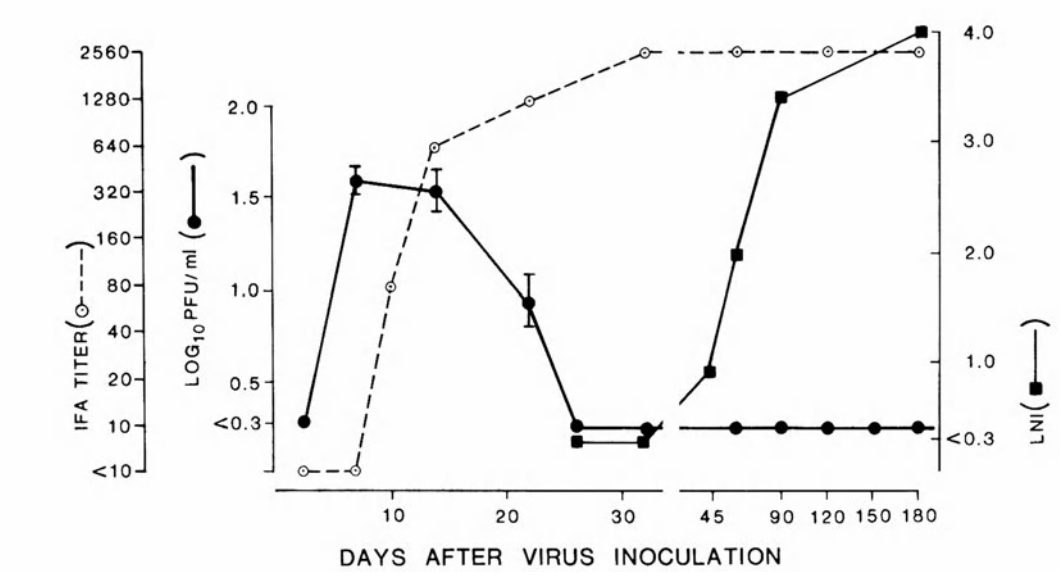

Fig. 1. Development of Lassa viremia (●), immunofluorescent (IFA) antibody (⊙), and neutralizing (■) antibody responses in four rhesus monkeys surviving s.c. inoculation of 6.1 $\log_{10}$ PFU Lassa virus (strain Josiah). Neutralizing antibody was measured in a plaque-reduction test in which 10% fresh normal monkey serum and 10% immune serum were incubated with ten fold virus dilutions for 1 h at 37°C. Residual virus was measured, and the $\log_{10}$ of the reduction in viral titer or log neutralization index (LNI) was calculated (JAHRLING et al. 1982)

that the early antibody response was not associated with reduction in viremia nor with recovery from disease. A comparison of viremias among surviving unmanipulated rhesus monkeys vs those that died illustrates a striking parallel with the human situation (MCCORMICK et al. 1986). Viremia titers in all lethally infected monkeys exceeded 4 $\log_{10}$ PFU/ml, while none of the surviving monkeys ever developed viremias in excess of this apparently critical titer.

Viremias and serologic response patterns typical of monkeys surviving Lassa infection are illustrated for four surviving rhesus monkeys (Fig. 1). Modest viremia titers peaked 7–11 days after inoculation and gradually subsided to undetectable levels by day 27. The serologic response as measured by IFAT was brisk, first detectable after 7–10 days and reaching maximum titers of 1280–2560 by day 33. In contrast, the neutralizing antibody response was undetectable before day 45, suggesting that neutralizing antibody was not critical to clearance of viremia. However, LNI titers continued to increase late into convalescence (90–180 days). A similar pattern has been described for antibody responses in human Lassa fever patients (JAHRLING et al. 1985b).

Further data from the unmanipulated primate models have included total and differential leukocyte counts, hemoglobin, SGOT, and CPK determinations. In general, fluctuations were mild in capuchin and squirrel monkeys and pronounced in the lethally infected species, except for hemoglobin, which did not fluctuate in any monkeys examined. SGOT and CPK concentrations were significantly elevated within 7 days of infection, and peaked by day 10 in monkeys destined to die.

Monkeys of all five species shed virus sporadically from the nasopharynx and in urine during the first 4 weeks of infection. Examination of tissues obtained from Lassa virus-infected monkeys when they were moribund or dead revealed significant viral replication, as measured by infectivity titration and immunofluorescence examination, in most visceral tissues tested. These included the lung, liver, adrenal gland, pancreatic acinar cells, splenic red pulp, kidney, and lymph nodes. In contrast, the brain stem, cerebellum, and spinal cord were largely spared, with the exception of structures resembling vascular endothelium.

Histopathological changes were mild compared with the extensive viral burden of visceral tissues. Focal hepatic and adrenal necrosis and interstitial pneumonitis were consistent findings (CALLIS et al. 1982; WALKER et al. 1982). The basic mechanism of pulmonary edema was also believed to be vascular damage and increased capillary permeability (Sect. 3.5). Adrenal glands frequently exhibited cortical necrosis, but occasionally cortical infarcts severe enough to cause adrenal failure were observed (CALLIS et al. 1982). Occasional ischemic infarcts, suggestive of vasculitis, were more severe in the Rhesus monkey model than in human Lassa fever.

Rhesus and cynomolgus monkeys have been utilized as models for human Lassa fever and as susceptible species for testing antiviral drugs, immunotherapy regimens, and candidate vaccines (JAHRLING et al. 1980; WALKER et al. 1982; JAHRLING et al. 1984).

2.3 Other Old World Arenaviruses

Several Old World arenaviruses and LCMV form the Old World arenavirus complex. Using antisera raised in guinea pigs, broadly cross-reactive relationships were observed by IFA among three Lassa virus strains plus Mopeia, Mobala, and LCM viruses. Heterologous IFA titers for the serum raised against LCMV were four to eight fold lower against Lassa, Mopeia, and Mobala viruses compared with LCMV. Conversely, the antiserum raised to Lassa, strain Josiah, had an eight fold lower titer against LCMV. The other antisera did not differentiate among the six viruses tested.

Table 2. Relationships among Old World arenaviruses by the neutralization test ($\log_{10}$ neutralization index determined by reduction of plaques)

Guinea pig immune serum			LNI of antiserum against virsus from					
Virus	Strain	Origin	SL	LIB/G	NIG	MOZ	CA	LCM
Lassa	Josiah	Sierra Leone (SL)	*3.6*	3.5	1.0	1.4	0.7	0.8
Lassa	Z-158	Liberia/Guinea (LIB/G)	2.6	*3.5*	1.5	1.5	1.2	0.4
Lassa	JW	Nigeria (NIG)	1.2	1.4	*3.6*	1.0	0.2	0.5
Mopeia	20410	Mozambique (MOZ)	<0.3	<0.3	<0.3	*2.4*	0.6	<0.3
Mobala	3080	Central Africa (CA)	<0.3	<0.3	<0.3	0.3	*2.2*	<0.3
LCM	Armstrong	USA	<0.3	<0.3	<0.3	<0.3	0.6	*3.9*

In contrast, these viruses were clearly distinguished by the same antisera in a plaque-reduction neutralization test (Table 2). LNI titers for homologous strains were invariably higher than for heterologous strains. Among the three West African Lassa viral strains, the Nigerian strain (JW) was the most distantly related. Antisera to these three strains had relatively high titers (LNI = 3.5–3.6 against homologous virus) and neutralized small but reproducible quantities of Mopeia, Mobala, and LCM viruses. The anti-LCMV serum was even more specific, with a high homologous titer (LNI = 3.0), minimal activity against Mobala (LNI = 0.6), and no neutralization of Mopeia or the three Lassa viral strains. Sera raised against Mopeia and Mobala were also specific, neutralizing only homologous strains, but with low LNI titers, thus precluding detection of potential low level cross-reactivity with the other viruses.

Comparisons among these strains by means of oligonucleotide "finger-printing," peptide analysis, and monoclonal antibody reactivities confirmed that they were related, yet distinct (GONZALES et al. 1984). Lassa, Mobala, and Mopeia shared 42%–75% homology in common oligonucleotides. By this method Mobala had more sequences in common with Lassa than Mopeia, in contrast to the biological similarities of Mopeia and Mobala. However, monoclonal antibody reactivities and peptide map comparisons clearly showed Mobala and Mopeia to be more closely related to one another than either was to Lassa. Monoclonal antibody techniques (CLEGG and LLOYD 1984; GONZALEZ et al. 1984) identified shared common antigenic sites in nucleoproteins, as well as glycoproteins; however, the three viruses can also be readily differentiated by monoclonal antibodies to each structural protein.

The pathogenicity of Mopeia or Mobala for man is unknown, but both viruses are benign for Lassa-susceptible species such as macaques and strain 13 guinea pigs. In contrast, Mopeia and Mobala viruses are lethal for outbred suckling ICR mice, unlike West African Lassa strains that are generally benign for them (GONZALEZ et al. 1984).

Mopeia causes an inapparent infection of rhesus monkeys characterized by seroconversion within 10 days and protection against subsequent challenge with a virulent Lassa virus strain (KILEY et al. 1979). It is not certain whether Mopeia causes histopathological lesions in rhesus monkeys or not. Mopeia-immunized monkeys necropsied after challenge with Lassa had hepatic, renal, and CNS lesions that could have been caused by either the immunizing or challenge virus (WALKER et al. 1982). More recently, a report on two monkeys killed while healthy 12 and 13 days after Mopeia inoculation described focal interstitial nephritis, focal hepatic necrosis with mononuclear cell infiltrates, and occasional hypertrophy of endothelial cells in the spleen (LANGE et al. 1985). In our laboratory we have compared the virological, immunological, and pathological events of Mopeia, Mobala, and virulent Lassa virus infections in eight rhesus monkeys for each strain, using colony-born and -raised primates. Neither Mopeia nor Mobala produced viremia, or significant fever, or significant fluctuations in RBC, WBC, Hb, hematocrit (HCT), SGOT, SGPT, CPK, latic dehydrogenase (LDH), or BUN. Lassa, in contrast, produced marked perturbations in all these parameters, and the monkeys died. Careful examination of all tissues, including kidney and liver from two clinically normal Mopeia-infected macaques killed

Table 3. Virulence of Mopeia virus (20410) for strain 13 guinea pigs and rhesus monkeys[a]

Virus	Animal	Age	Dose (log_{10} PFU)	Route	Dead/ inoculated	Days of death
Mopeia	Strain 13 guinea pig	3-5 months	6.1	s.c.	0/10	–
			4.1		0/25	–
			1.1		0/10	–
			4.9	i.n.	1/10	18[b]
		5-7 days	4.1	s.c.	0/10	
			4.9	i.n.	0/10	
			3.9	i.c.	2/10	2,4[b,c]
	Rhesus monkey	adult	4.1	s.c.	1/6	13[d]
Isolate from PAX-180[d]	Guinea pig	3 months	3.6	s.c.	0/9	–
Isolate from PAX-32[e]	Guinea pig	3 months	3.1	s.c.	0/5	–

[a] Viremia <0.3 log_{10} PFU/ml for all animals tested on days 7, 14, and 21. All animals surviving to day 21 seroconverted according to IFAT ($\geqq 20$)

[b] Virus isolation attempt negative (blood, lung, spleen, kidney, adrenal, brain)

[c] Early deaths attributed to trauma of i.c. inoculation

[d] Monkey PAX-180 died on day 13 from aspiration of vomit while under anesthesia. No histopathologic lesions were found. Virus isolation attempts were negative except for urine aspirated from bladder (1.7 log_{10} PFU/ml)

[e] Monkey PAX-32 was apparently healthy and had no histopathological lesions when killed on day 167. Virus was obtained only from spleen explant and was identified as Mopeia by neutralization

on day 13, revealed no lesions distinct from background. We did find, however, significant shedding of virus in the urine of Mopeia-infected rhesus monkeys from day 7 through day 20. This virus was benign on passage to strain 13 guinea pigs, providing some reassurance that Mopeia does not increase in virulence after passage in primates.

Mopeia virus was also rescued by cocultivation of Vero cells with spleen cells from a convalescent rhesus monkey (PAX-32) 167 days after inoculation (Table 3). This virus was identified as Mopeia in a cross-neutralization test, and was demonstrably benign for strain 13 guinea pigs. Such prolonged latency of a rodent arenavirus in a primate host may not be unusual, since we have regularly used the same techniques to recover Lassa virus from surviving macaques 6 weeks to 2 years after infection.

We studied the pathogenesis of Mopeia for strain 13 guinea pigs in more detail (Table 3). None of the adult guinea pigs developed fever, viremia, or fluctuations in hematologic parameters or blood chemistry after s.c. or intranasal (i.n.) inoculation. The one guinea pig that died following i.n. infection failed to yield virus from any visceral organ, including the brain, suggesting that death was not related to viral exposure. Even infant (5- to 7-day-old) guinea pigs resisted s.c., i.n., and i.c. challenge, and failed to attain detectable viremia. Two infant guinea pigs died soon after i.c. challenge (on days 2 and 4), but the deaths were attributed to trauma of i.c. inoculation, since virus isolation attempts were again negative. All guinea pigs seroconverted according to IFAT

within 20 days, although neutralizing antibody responses were markedly delayed (data not shown). Similar data were obtained with adult strain 13 guinea pigs inoculated s.c. with 4.0 $\log_{10}$ PFU of Mobala.

2.3.1 Cross-Protective Relationships Among Old World Arenaviruses

Guinea pigs infected with Lassa virus developed homologous N antibodies, but other Old World arenaviruses induced little or no serum N response to Lassa virus. Nevertheless, all were protected against virulent Lassa challenge, even those immunized with LCMV ARM (Table 4). If LCMV WE was used as a test virus in LNI, titers were low except in sera from LCMV ARM-infected animals. Prior infection with LCMV ARM or Lassa virus protected against lethal LCMV WE challenge; however, neither Mopeia nor Mobala protected LCMV WE-challenged animals. Thus, specific cross-protective relations were observed, but N antibody did not predict the patterns.

To examine further the basis for cross-protection, we performed cytotoxicity studies with spleen cells from guinea pigs inoculated with Lassa, Mopeia, or LCMV ARM, using syngeneic guinea pig kidney (GPK) target cells (Table 5). Spleen cells from guinea pigs infected with Lassa virus Z-158 produced 45%–53% lysis of GPK targets infected with any of the four viruses tested, including LCMV. In contrast, Mopeia-immunized spleen cells lysed Lassa- or Mopeia-infected targets but not LCMV-infected ones. Likewise, LCMV-immune spleen cells recognized Lassa- and LCMV-infected targets but not Mopeia-infected ones. These patterns are concordant with the cross-protection data (Table 4) and suggest that cytotoxic spleen cells may be important effectors of cross-protection. In preliminary studies similar immunologic relationships were established among these viruses in a cytotoxicity assay which employed murine spleen cells from C57BL6/J mice and MC-57 cell targets (G.A. Cole, pers. comm.).

Table 4. Cross-protection between Lassa virus strains and LCMV in strain 13 guinea pigs

	Results of challenge[a] in guinea pigs			
	Lassa (Josiah)		LCMV (WE)	
Immunizing strain	LNI[c]	Dead/total	LNI	Dead/total
Lassa (Josiah)[b]	3.9	0/25	1.5	0/10
Lassa (Z-158)	3.7	0/10	0.3	0/12
Mopeia	0.2	0/28	0.0	25/25
Mobala	0.4	0/10	0.4	10/10
LCM (ARM)	0.0	0/20	3.1	0/25
None	0.0	50/50	0.0	20/20

[a] Guinea pigs challenged s.c. with 3.7 $\log_{10}$ PFU Lassa virus or 4.1 $\log_{10}$ PFU LCMV 75-90 days after an immunizing infection

[b] Protected by treatment with ribavirin

[c] $\log_{10}$ neutralization index against the challenge viral strain

Table 5. Lysis of virus-infected target cells by effector strain 13 guinea pig spleen cells (from JAHRLING and PETERS 1986)

Effector spleen cells (E:T = 50)	Percent lysis of GPK targets[a]			
	Lassa (Josiah)	Lassa (Z-158)	Mopeia	LCMV (WE)
Lassa (Z-158)	48	51	45	53
Mopeia	42	39	46	3
LCM (ARM)	45	36	6	58

[a] Spleen cells harvested from stain 13 guinea pigs infected 15 days previously were tested on ^{51}Cr-labeled GPK targets in an 18 h assay. GPK is a cell line derived from the kidneys of a strain 13 guinea pig from our colony. As controls, effector spleen cells from immunized guinea pigs failed to lyse uninfected GPK targets; likewise, control (unimmunized) spleen cells failed to lyse virus-infected GPK targets

Table 6. Cross-protection by spleen cells or plasma from immunized strain 13 guinea pigs

Immune donors		Results of challenge in recipients (recipients dead/5 inoculated)[a]			
		Lassa (Josiah)[b]		LCMV (WE)	
Virus	Days	Cells	Plasma	Cells	Plasma
Lassa (Z-158)[b]	15	0	5	0	5
Mopeia	15	0	5	5	5
LCM (ARM)	22	0	5	1	5
Lassa (Z158)	55	4	1	5	5
Mopeia	47	4	5	5	5
LCM (ARM)	57	5	5	5	1

[a] Recipients were given 1.5×10^8 nucleated spleen cells (approximately 1 spleen-equivalent) i.p. on the day of challenge or 3 ml/kg plasma i.p. on each of days 0, 3, and 6

[b] The Josiah strain of Lassa virus is lethal for strain 13 guinea pigs, while the Z-158 strain is not; the former was used as a lethal challenge virus and the latter as an immunizing virus

We also performed passive protection experiments to evaluate the in vivo role of spleen cells (Table 6). Transfer of the equivalent of one spleen from inbred strain 13 guinea pigs infected 15–22 days earlier with Lassa, Mopeia, or LCMV completely protected against virulent Lassa challenge. When LCMV WE was the challenge virus, Lassa- or LCMV-primed cells spared the recipients, whereas cells from Mopeia-infected animals had no activity. Plasma from the spleen cell donors had high IFA titers, no detectable N antibody and, as expected, did not protect.

Interestingly, spleen cells harvested later after infection (47–57 days) had little or no protective activity even against homologous challenge. Whatever

the mechanism, lymphoid cells taken late after infection have also been found to be inefficient in controlling viral replication and inducing immunopathologic encephalitis in the LCMV-infected mouse (JOHNSON and COLE 1975). By this time Lassa- and LCMV-infected donors had homologous N antibodies, and plasma infusion could confer protection.

These adoptive transfer experiments support the hypothesis that effector spleen cells are important in recovery from Lassa or LCMV infection and that cross-protection may be mediated by these cells in the absence of cross-reactive neutralizing antibody. Furthermore, they furnish an example of a circumstance where humoral antibody as measured by the virus N test can be utilized in protection, an application discussed in detail in Sect. 5.2.

2.4 Pichinde Virus

2.4.1 Hamster

Pichinde virus is a member of the New World arenavirus complex; it is serologically related to, yet clearly distinguishable from Junin and Machupo viruses. Pichinde virus is not a human pathogen; however, the virus produces an infection in MHA hamsters which simulates human Lassa fever (BUCHMEIER and RAWLS 1977; MURPHY et al. 1977). The MHA hamster-Pichinde model was among the first to provide insight into the pathogenesis of hemorrhagic fever in human arenavirus infections. In contrast with MHA hamsters, outbred LVG and inbred LSH strain hamsters are resistant to Pichinde infection. Comparison of the virological and immunological events in resistant and susceptible hamsters infected with Pichinde has suggested that cellular injury and disease are direct viral effects and are not immunologically mediated. As in human arenavirus hemorrhagic fever syndromes the immune response appears to serve only a protective role.

MHA hamsters were uniformly infected and killed by peripheral (s.c. or i.p.) inoculation of 3–5 $\log_{10}$ PFU of Pichinde virus. Peak viremia titers of 8–9 $\log_{10}$ PFU/ml were reached within 8 days and were maintained until all animals died, 10–14 days after inoculation. In contrast, resistant LVG hamsters developed peak viremia titers of only 2–3 $\log_{10}$ PFU/ml which cleared by day 15. Moribund animals had significantly elevated neutrophil counts (14000–17000 cells/ml) with a pronounced shift to the left and slightly reduced HCT. Typical findings in the urine included protein and hyaline casts and hematuria; also, in contrast to the normally alkaline pH of hamster urine, infected animals had acidic urine. Serum enzyme determinations have not been reported.

Viral infectivity titrations identified spleen, liver, and kidney (but not brain) as major sites of viral replication. In MHA hamsters infectivity titers were always at least 100-fold greater than in tissues of resistant LVG hamsters. However, no inherent differences were found between MHA and LVG hamsters in their abilities to support viral replication, as assessed in primary kidney or peritoneal exudate cells (BUCHMEIER and RAWLS 1977). When CY was used

to produce a generalized immunosuppression, LVG hamsters lost their resistance and their ability to limit viral replication in target tissues. Thus, resistance appears to depend on an immunological control of viral replication.

Histopathological examination of tissues from Pichinde-infected hamsters confirmed that spleen, liver, and kidney were major target organs (MURPHY et al. 1977). A critical virological activity appeared to be the invasion of reticuloendothelial cells. The degree of histopathological injury in MHA hamsters was more severe than that reported for most other lethal arenavirus infections in animal models.

In the spleens of moribund animals, necrosis of red pulp and destruction of cord-sinus architecture were widespread. In the liver 20%–30% of hepatocytes were necrotic, and variable numbers of Kupffer cells were involved; however, no inflammatory cells were observed. Moderate amounts of renal tubular necrosis and calcifications were observed in the outer zone of the medulla. In addition, viral antigen was detected in occasional bone marrow cells thought to be macrophages and in approxiomately 10% of the megakaryocytes. Viral antigen was also found in the meninges and epithelium of the choroid plexuses of MHA hamsters. Viral antigen was generally found in and around blood vessel walls.

The immunological mechanisms that control Pichinde infection in resistant LVG hamsters and that are apparently deficient in MHA hamsters are not understood. Complement-fixing antibodies do not appear to play a major role, since they appear at about the same time (10–14 days) in both hamster strains, as do antibodies to viral antigens at the cell surface (BUCHMEIER and RAWLS 1977) and immunofluorescent antibodies (JAHRLING, unpublished observation). Likewise, neutralizing antibody titers in LVG hamsters evolve long after viremia has cleared (JAHRLING, unpublished observation), suggesting that N antibody is not involved in recovery from primary infection. The critical immunological response is CY sensitive, since CY immunosuppression rendered LVG hamsters susceptible to lethal infection. However, direct evidence linking an immunologic response to resistance is sparse. The increased susceptibility of MHA hamsters may be related to their failure to mount a delayed hypersensitivity response (as measured by footpad swelling after inoculation by this route), in contrast to LSH and LVG hamsters, which develop footpad responses within 8 days of inoculation (GEE et al. 1981). Another clue for explaining differences in susceptibility comes from observed differences in NK-cell activity among hamster strains (GEE et al. 1979). Spleen cells from infected MHA hamsters that copurified with NK-cell activity were shown to contain ten times the number of infectious centers than a comparable population from LSH hamsters (GEE et al. 1981). Genetic control of low NK-cell activity appeared to be dominant in hamsters, and both survival and ability to limit viremia segregated in a 1:1 ratio in backcross experiments. However, it is not yet proven that the same gene controls NK-cell activity and survival.

The virological, immunological, and histopathological nature of lethal Pinchinde infection in MHA hamsters is remarkably similar to Lassa virus infection in animal models. More precise elucidation of the critical immunological mechanisms responsible for resistance of LVG or LSH hamsters might provide valuable insight into host resistance to human arenavirus infections in general.

2.4.2 Guinea Pig

Pichinde virus was adapted to produce uniformly lethal infections of strain 13 guinea pigs by sequentially passaging the virus in guinea pig spleens (JAHRLING et al. 1981). Adapted Pichinde virus (passage 8) was not pathogenic for rhesus monkeys, outbred Syrian hamsters, and adult ICR mice, and like parental virus, is considered safe to use at a minimal (P–2) biocontainment level.

All inbred strain 13 guinea pigs died within 13–19 days after s.c. inoculation of 3 PFU or more of adapted Pichinde. Infected guinea pigs developed a viremia $>5 \log_{10}$ PFU, lymphopenia ($<1000/mm^3$), neutrophilia ($>6000/mm^3$), and moderate elevations in serum transaminase levels.

Histologically, there were numerous, randomly distributed foci of hepatocellular necrosis and diffuse fatty change in the liver. Pancreatic acinar tissue was atrophic and acinar cells contained numerous cytoplasmic vacuoles. There was significant interstitial pneumonia. The spleen and adrenal cortices often were congested and contained small necrotic foci. Pichinde antigen distribution corresponded to these histopathological lesions, but with more extensive involvement. Except for the presence of Pichinde antigens in occasional vascular endothelial cells, there was no evidence of CNS invasion.

Thus, infection of strain 13 guinea pigs with adapted Pichinde is similar in many respects to Lassa fever in humans and provides a model to study the physiology, pathogenesis, immunology, and therapy of arenavirus HF without the constraints of P–4 containment. Furthermore, the virus and host interactions determining outcome of infection can be studied by comparison of the immunological and virological events in strain 13 guinea pigs comparing parental vs adapted virus strains, or by comparing the process in outbred Hartley vs strain 13 guinea pigs, infected with adapted Pichinde virus.

2.5 Machupo Virus

2.5.1 Guinea Pig

Infection of guinea pigs with prototype Carvallo strain of Machupo virus yields highly variable results. Lethality ranges from 20%–80% and is essentially independent of virus dose over a wide range (EDDY, unpublished observations). However, after five spleen-spleen 14–day harvests, uniform mortality was observed following administration of as little as 2 PFU. Gross and microscopic pathological changes in guinea pigs after infection with Machupo are similar to those reported following Junin viral infection (EDDY, unpublished observations).

2.5.2 Monkey

Many New World primates, including *Aotus* and *Cebus* spp., have not been susceptible to induction of clinical disease after infection with Machupo virus.

However, Central American marmosets (*Saguinus geoffroyi*) infected s.c. or by scarification with hamster brain suspensions of Carvallo strain developed a progressive, reproducible illness lethal within 8–20 days. Findings included anorexia, lethargy, weakness, tremors, and clinical shock, which were observed beginning around 3 days prior to death. Virus could be isolated from throat swabs, urine, blood, spleen, kidneys, heart, brain, and liver. There were no hemorrhagic manifestations, but cortical necrosis of lymph nodes and splenic reticular hyperplasia with lymphoid depletion were described (WEBB et al. 1975).

Infection of Old World primates (cynomolgus and rhesus macaques, and African green monkeys) with Machupo virus resulted in a distinctive biphasic illness with an initial viremic phase and subsequent progressive neurological deterioration (KASTELLO et al. 1976; EDDY et al. 1975a; EDDY et al. 1975b; WAGNER et al. 1977; SCOTT et al. 1978; MCLEOD et al. 1978). In rhesus macaques, the most throughly studied species, s.c. inoculation of 3.0 $\log_{10}$ PFU of the Carvallo strain resulted in anorexia, lassitude, gastrointestinal disturbances, and conjunctivitis. Erythematous facial and abdominal rashes were observed frequently, and a nasal discharge, which occasionally became hemorrhagic, occurred in half the animals. In most macaques progressive lethargy, cutaneous hemorrhage, dehydration, and weight loss terminated in hypotensive shock and death 25–30 days after inoculation. During this acute stage of illness, platelets, HCT, lymphocyte, and neutrophil values fell progressively until just before death.

Viremia was detected by day 5 postinfection and persisted for the duration of the acute illness, with peak titers reaching 5–6 $\log_{10}$ PFU/ml within 2 weeks. Neutralizing antibodies did not develop in animals that died during this stage of the disease. Histological findings included modest degrees of hepatic necrosis with Councilman-like bodies, necrotizing enteritis, epithelial necrosis, and adrenal cortical necrosis. Myocardial degenerative lesions, lymphoid depletion, lymphoid and reticuleondothelial cell hyperplasia, and hemorrhage were frequent, but inconstant, findings. Animals succumbing after 17 days demonstrated a nonsuppurative meningoencephalitis with vasculitis and perivascular cuffs (TERRELL et al. 1973).

About 20% of the monkeys, particularly larger, more mature animals, began to improve 3 weeks postinoculation. Activity and appetite were enhanced and fluid status restored; however, between 26 and 40 days postinfection, most of these early survivors developed neurological signs, with severe intention tremors, nystagmus, paresis, ataxia, and coma. This late neurological syndrome was relentless, and survival rare. Viremia was not detectable in monkeys during the neurological phase of illness, and neutralizing antibodies were universally present by day 28 in all animals destined to survive the early (hemorrhagic) phase. Monkeys that developed late neurological illness showed widespread perivascular and diffuse mononuclear cell infiltrates in abdominal viscera, parathyroid glands, and heart. Lymphocytic inflammation and vasculitis were present throughout the brain and spinal cord. Ganglia and peripheral nerves were diffusely infiltrated with lymphocytes as well (MCLEOD et al. 1976).

2.6 Junin Virus

2.6.1 Guinea Pig

The guinea pig has been the most consistently employed experimental model for infection with Junin virus. Most of the information concerning this model derives from infection with the prototype XJ strain, originally isolated from a fatal human case of AHF (MOLINAS et al. 1978; CARBALLAL et al. 1977; OUBINA et al. 1984; CARBALLAL et al. 1981; FRIGERIO 1977). When guinea pigs are inoculated i.m. with the XJ strain, they develop fever and weight loss starting about 7 days after infection and shortly thereafter develop leukopenia and thrombocytopenia. All infected animals die with a hemorrhagic diathesis by 13–18 days after infection. By fluorescent antibody, ultrastructural, and direct virus isolation techniques, virus appears to replicate predominantly in the spleen, lymph nodes, and bone marrow, where major histological evidence of damage occurs.

Availability of additional, naturally occurring strains afforded us the opportunity for comparing disease patterns in guinea pigs inoculated with low-passage-level isolates. A broad spectrum of virulence was observed in animals infected with these viral strains, which was independent of host background (outbred Hartley strain or inbred strains 2 and 13). Some Junin strains required less than 1 PFU to produce an LD_{50}, while other strains killed about 20% of infected guinea pigs, regardless of dose inoculated (Table 7).

We observed two predominant disease forms which we term visceral and neurological (Table 8). Visceral disease resembles that described for the prototype XJ strain. Typically, we showed that virus replicated predominantly in spleen, lymph nodes, and bone marrow by 5–6 days postinfection. At death, by days 13–17, there were 5–6 $\log_{10}$ PFU/g in these tissues. Lower levels of

Table 7. Virulence of Junin viral strains for outbred guinea pigs

Strain[a]	$\log_{10}$ PFU/LD_{50}	Mean time to death ±S.D. (days)[b]	Guinea pig disease pattern
Espindola	−1.3	17.3±2.7	hemorrhagic
Romero	−0.7	14.5±1.7	hemorrhagic
Ledesma	−0.5	19.0±2.3	hemorrhagic/neurological
3551	2.2	21.1±2.5	neurological/hemorrhagic
Suarez	4.0	27.8±3.9	neurological
Coronel	[c]	30	neurological
XJ-44	>7.0	–	none
Candid 1	>7.0	–	none

[a] The first six virus strains were isolated from patients with AHF; XJ44 is a laboratory-derived attenuated virus and Candid 1, an AHF vaccine candidate

[b] Outbred guinea pigs inoculated i.p. with 100 LD_{50}, except for Coronel strain, which received 5.0 $\log_{10}$ PFU

[c] Approximately 20% died at virus doses between 1 and 6 $\log_{10}$ PFU. Strain 13 guinea pigs were not significantly more susceptible

Table 8. Predominant disease forms in Junin virus-infected guinea pigs

Disease manifestations	Time to death (days)	Predominant clinical sign	Predominant lesion	Predominant virus isolation
Hemorrhagic	10-17	Weight loss	Spleen and bone marrow necrosis	Spleen, lymph node, bone marrow
Neurological	15-30	Hind-limb paralysis	Encephalitis	Brain

virus were generally found in blood, and virus in brain homogenates usually approximated that found in blood. Predominant pathological lesions included necrosis and depletion of cells in the spleen, lymph nodes, and bone marrow. In contrast, animals developing the neurological form (generally manifested as a progressive hind-limb paralysis) displayed low levels of virus in the spleen and lymph nodes on days 5–7; by days 10–13, however, virus was no longer detectable in these animals. Between days 20 and 30, virus could be recovered from brain tissue, attaining titers of 5 $\log_{10}$ PFU or greater after day 25. Low levels of circulating antibody were often detectable in these animals by the time of onset of paralysis. Brain tissues from the paralyzed animals generally showed moderate polioencephalitis.

Of interest is the finding that the route of infection of guinea pigs with human-virulent strains of Junin virus appears to have little effect on the final outcome or on the clinical course of the disease. Even when inoculated i.c. with Romero strain (a strain highly virulent for guinea pigs), the clinical disease pattern was indistinguishable from that following i.p. or s.c. inoculation. However, when we inoculated attenuated XJ44 strain into the brain, 17 of 22 guinea pigs died with a neurological form of the disease and with 3–4 $\log_{10}$ PFU of virus in the brain. None of the guinea pigs inoculated i.p. or s.c. with this strain died or showed clinical abnormalities.

2.6.2 Monkey

Primates of both Old and New World origin have been examined as potential models for Junin viral disease. Among species studied to date, rhesus macaques (*Macaca mulatta*) have demonstrated clinical, pathological, and laboratory findings most closely approximating those of humans with AHF (McKee et al. 1985b, 1986; Green et al. 1986). Beginning 7–8 days after i.m. inoculation with 4–5 $\log_{10}$ PFU of Espindola strain Junin virus, animals developed anorexia, lassitude, and gastrointestinal disturbances. Within 7–10 days, prominent facial flushing, a macular rash (distributed across the face, axillae, and upper arms), purulent conjunctivitis, and oral ulcerations were seen. As the disease progressed, infected monkeys developed a pronounced hemorrhagic diathesis, with widespread petechiae and mucous membrane bleeding. All animals ultimately succumbed; the mean time to death was 33 days. Terminally, dehydration and cachexia with profound weight loss (up to 25% of preinoculation values) were seen.

The Espindola virus isolate was recovered from a dying patient whose disease had been classified as a "hemorrhagic" form of AHF. When additional low-passage isolates of Junin virus obtained from humans with each of the other clinically recognized variants of AHF ("neurologic", "mixed", and "common" forms) were studied, patterns of clinical illness were found to be virus strain-specific. Each isolate induced a disease syndrome in monkeys that faithfully replicated the clinical variant of the disease in the human from whom the viral strain was obtained.

In animals infected with Romero, an isolate recovered from a patient with a nonfatal "common" disease variant, the initial nonspecific findings observed in macaques were seen, but disease signs were transitory, and spontaneous recovery occurred in all cases within 10 days. The Ledesma strain of Junin virus was isolated from a terminally ill patient with the "neurologic" form of AHF. Although initial signs of disease resembled those seen in macaques infected with other viral strains, within 1–2 weeks the clinical picture was dominated by neurological involvement (tremulousness, hyperactive startle reflexes, ataxia, and limb paresis).

Clinicopathological and histopathological studies support clinical impressions of virus-specific disease patterns. Transient depression of total white blood counts occurred between days 7 and 10 postinfection in Romero-, Espindola-, and Ledesma-infected animals; lymphocytes and granulocytes were equally affected. Anemia was a feature only among Espindola-infected macaques, occurring preterminally. Platelet counts remained normal during clinically benign Romero infections, but fell dramatically in Espindola- and Ledesma-infected monkeys to less than 100000/mm^3 in most animals by the end of the 3rd week.

At necropsy monkeys in the Espindola group demonstrated diffuse hemorrhages, lymphocyte depletion of lymph nodes and spleen, bone marrow necrosis, mild hepatocellular necrosis, and minimal to mild CNS lesions which developed only in those animals surviving longest. In contrast, CNS lesions developed much earlier and were of markedly greater severity among Ledesma-infected macaques. While lymphocyte depletion was prominent, hepatocellular necrosis, bone marrow necrosis, and hemorrhages were very mild or absent in these animals.

In monkeys infected with both Espindola and Ledesma, inflammatory and degenerative changes within autonomic ganglia were found. It is tempting to speculate that these lesions represent pathologic correlates of viral involvement of the autonomic nervous system. Such lesions could explain the hypotension, flushing, or gastrointestinal disturbances so characteristic of this disease.

Viremia was undetectable in Romero infection. With Ledesma, serum viral titers were consistently less than 3 $\log_{10}$ PFU/ml and of brief duration, while in Espindola-infected animals, pronounced serum viremias (generally 5–7 $\log_{10}$ PFU/ml) were observed that frequently persisted at titers exceeding 3 $\log_{10}$ PFU/ml until death. Serum antibody responses varied with viral strain as well. Immunofluorescent and neutralizing antibodies were detected in Romero- and Ledesma-infected animals during the 2nd postinfection week. Serological responses among Espindola-infected monkeys were delayed by a week or more, however, and frequently were not measurable by the time of death.

Infection of rhesus macaques with Junin virus thus provides a reasonably accurate model for human AHF with the additional bonus of demonstrating the role of virus-determined factors in the pattern of biological response in this system.

Among New World primates, *Callithrix jacchus* has proved to be a useful model for study of Junin viral disease (WEISSENBACHER et al. 1979; GONZALEZ et al. 1983). This small neotropical marmoset manifests severe and reproducible disease after infection with prototype XJ strain. Inoculated animals developed anorexia, weight loss, hemorrhages, hyperexcitability, and tremors, together with anemia, diminished leukocyte and platelet counts, and viremia. Histopathological changes included hepatocellular necrosis, lymphocytic depletion within spleen and lymph nodes, interstitial pneumonitis, and a diffuse meningoencephalitis of late onset and variable intensity.

Experimental infections of other New World species have been less rewarding. *Cebus* sp. (capuchin) monkeys infected with XJ (CARBALLAL et al. 1983) or other (MCKEE et al. 1985a) Junin viral strains developed mild or inapparent clinical disease with mild and inconstant neuropathological sequelae. *Alouetta caraya* (howler monkeys), *Saimiri sciureus* (squirrel monkeys), and *Aotus trivirgatus* (owl monkeys) (FRIGERIO et al. 1982) infected experimentally with the XJ strain underwent seroconversion without overt clinical illness.

2.7 Aerosol Infections

The airborne infectivity of arenaviruses is attested to by numerous anecdotes of laboratory infections. For example, a dramatic instance of aerosol transmission of LCMV occurred in our laboratory when eight cynomolgus monkeys were temporarily housed in a small room which also contained several cages of C57Bl/6 strain mice vertically infected with LCMV WE. Ten days later the monkeys were moved to an LCMV-free room. Within the next 5 days six of the eight monkeys died, and LCMV was isolated from the blood and visceral tissues of all six. The remaining two monkeys did not become ill, nor did they seroconvert to LCMV. Minute quantities of airborne LCMV were detected in the room air collected by an electrostatic precipitator which samples 1000 liter/min, but only during routine changing of the mouse pan bedding. Presumably the monkeys were exposed during one of these manipulations.

Lassa virus was also inadvertently transmitted, both from rodents to monkeys and between guinea pig cages. Sentinel strain 13 guinea pigs in cages interspersed in racks of Lassa virus-infected guinea pigs occasionally became fatally infected with Lassa virus, although inapparent serocenversions were not observed. Before the aerosol transmissibility of Lassa virus was fully appreciated, we often conditioned uninfected monkeys in the single room available for animal inoculation. On one occasion 12 cynomolgus macaques were introduced when guinea pigs and mice had been infected with a virulent Lassa viral strain 12 days previously. Two of these monkeys became infected and died within 3 weeks, yielding Lassa virus from all organs tested. Three of several hundred monkeys studied over the course of 5 years have also seroconverted without developing

overt disease while being conditioned before inoculation with Lassa virus. Transmission of virus between monkeys is possible; urine is frequently infectious, even late into convalescence. However, infection of monkeys in our experience has always occurred in the presence of Lassa virus-infected guinea pigs or mice. Large volume air sampling has not yielded Lassa virus, even during cage-changing operations, but dust generated from infected bedding is still considered a likely source of the infections.

Intercage transmission between Machupo virus-infected guinea pigs has also occurred, but stopped when filtered cages were used and bedding was moistened with disinfectant before cage cleaning. Transmission between cages of Junin virus-infected guinea pigs, which were maintained under similar conditions, did not occur. With animals maintained in laminar flow isolators, we observed transmission of the Espindola strain of Junin virus from inoculated rhesus monkeys to controls in adjacent cages; however, transmission has not occurred between isolators. Ledesma strain-inoculated macaques did not transmit AHF under these conditions. The critical variable may be the high concentration of Espindola strain found in the saliva of infected macaques or their more profuse hemorrhages.

These incidents emphasize both the potential hazards of airborne infection and the difficulties in critically analyzing such situations. The probability of aerosol dissemination can be reduced to a function of three variables: (1) whether the virus is actually aerosolized by the infected host or a secondary aerosol is generated from animal bedding or other source, (2) the stability of the virus once aerosolized, and (3) the infectivity of small-particle aerosols for the animal at risk.

Most studies have focused on quantitating the minimal infectious aerosol dose. For example, cynomolgus monkeys exposed to aerosol doses of several hundred mouse i.c. (MIC) LD_{50} of LCMV WE all died, while lower doses were less infectious and produced some sublethal infections. Rhesus monkeys were more sensitive, and were routinely killed by doses of 1 $MICLD_{50}$. Even lower doses produced some infections, although no deaths. In both species of macaque, deaths occurred 12–23 days after exposure. The lungs were shown to be infected early in the disease course, followed by a systemic infection indistinguishable from that following parenteral inoculation (DANES et al. 1963).

The aerosol infectivity of Lassa virus (Josiah strain) in cynomolgus monkeys was established by exposing animals to aerosols generated by a Henderson transit tube modified for animal exposure (STEPHENSON et al. 1984). Macaques were exposed to graded doses of virus-containing particles 4.5 μm in diameter for 10 min. Inhaled doses ranged from 2.7–4.5 $\log_{10}$ PFU, but all monkeys died, with a mean time to death of 14 days regardless of the virus dose. Viremia and fever evolution, also independent of dose, were similar to those reported for cynomolgus monkeys inoculated s.c. (JAHRLING et al. 1984). Histological lesions included mild, multifocal pneumonitis, hepatitis, myocarditis, and choroiditis, similar in intensity and distribution to that seen in monkeys inoculated s.c.

The median infectious dose of Lassa virus for outbred guinea pigs was found to be 15 PFU. About half of the infected animals died, similar to the mortality rate of s.c. inoculated outbred guinea pigs. Early replication of virus

occurred in the lungs and upper respiratory tract, as well as the spleen. As in s.c. inoculated animals, brain and liver were not major targets. Doses of less than 100 PFU were also lethal when Lassa virus was administered i.n. to anesthetized strain 13 guinea pigs (JAHRLING, unpublished observations).

Other Old World arenaviruses are also infectious for strain 13 guinea pigs inoculated i.n. Both Mopeia and Mobala uniformly infected guinea pigs exposed to 2.0–4.9 $\log_{10}$ PFU but produced inapparent infections, evidenced only by seroconversion (JAHRLING, unpublished observation).

Junin virus infects guinea pigs exposed i.n.; 4.5 $\log_{10}$ $TCID_{50}$ of strain XJ produced 100% infection and 100% mortality, while 3.5 $\log_{10}$ $TCID_{50}$ produced 100% infection and 83% mortality. The i.n. inoculation of the attenuated strain, XJ clone 3, produced 100% infection and 25% mortality at a dose of 6.5 $\log_{10}$ $TCID_{50}$ and 66% infection with no mortality at 3.5 $\log_{10}$ $TCID_{50}$ (SAMOILOVICH et al. 1983).

The aerosol stability of these viruses appears to be high. Formal studies with Lassa virus indicate a biological half-life of 55 min at 25°C and 30% relative humidity or 18 min at 25°C and 80% relative humidity (STEPHENSON et al. 1984). This degree of stability suggests that this virus poses a significant potential aerosol hazard if infected *Mastomys* rodents aerosolize their virus-laden urine, and in the hospital and laboratory settings, where numerous opportunities exist to generate aerosols from infectious body fluids and cell-culture supernatants. The greater stability at lower relative humidity is characteristic of many lipid-enveloped viruses in aerosol suspension and may have some relation to the greater number of Lassa fever cases observed during the dry season in West Africa.

Thus, any assessment of the role of airborne infection in the biology of arenaviruses must begin with the established aerosol stability of these viruses and their high aerosol infectivity in all HF models studied. It seems likely that humans, too, are susceptible to aerosol infection, since several episodes involving LCMV (reviewed in PETERS 1984), Lassa virus (WHITE 1972; MONATH and CASALS 1975), AHF (MAIZTEGUI, quoted in PETERS 1984), and BHF (PETERS et al. 1974) were all likely to have resulted from airborne spread. Thus, the opportunity for human infection by this route may depend largely on circumstances when small-particle aerosols can be generated. Transmission from rodent to human may involve aerosols quite frequently; indeed, field epidemiological observations suggest that many, if not most, cases of AHF are spread by aerosols. In contrast, interhuman transmission of the arenaviral HF is not common. Nosocomial dissemination of the South American HF has been reported in only a single instance, and there are only a handful of recognized episodes with Lassa virus. It seems likely that these unusual cases excrete virus (some in the form of aerosols) in unusual amounts, either because of patient or viral idiosyncracy. Emphasis on the low frequency of dissemination of Lassa virus (FISHER-HOCH et al. 1985a) is important for establishing a proper perspective on the care of cases occurring in endemic areas or outside West Africa. Nevertheless, the potential for aerosol transmission and the occasional episodes that have occurred must be considered when evaluating the hazard of transport, laboratory work, and medical attention for these patients.

2.8 Summary

Several infections of experimental animals mimic the important features of human arenaviral HF. Verisimilitude, host biology, cost, and biohazard vary, but strain 13 guinea pigs and macaques provide the rodent and primate systems of broadest utility.

In these model systems, as in humans, infections typically result in a viremia, which lasts the duration of the acute illness. Viral replication occurs in most organs, particularly the lymphoid system, liver, lung, kidney, and adrenals. Viral antigen is found in parenchymal cells of these organs, extensively in macrophages, and occasionally in cells resembling vascular endothelium.

Inflammation is minimal in most cases, although Lassa-infected squirrel monkeys and macaques, late in South American hemorrhagic fever infections, may have impressive perivascular cuffing. Necrosis is focal and not usually extensive. The disparity between extensive infection and minor histopathology suggests that viral infection itself may in some situations be responsible for cellular dysfunction without morphological changes (OLDSTONE 1984). The most constant host response to infection in these models is weight loss, which is an early and sensitive indicator of disease. Signs of increased vascular permeability, including edema and effusions, are frequently seen.

Two distinct patterns emerge. One resembles human Lassa fever and is seen primarily in Lassa, LCM, and Pichinde viral infections. The other is more characteristic of South American HF and is exemplified by Junin and Machupo infections of guinea pigs and susceptible primates. In Lassa-like infections significant virus-neutralizing antibody appears long after recovery and is never very efficient. In Junin or Machupo infections virus-neutralizing antibodies have a closer temporal relationship to recovery and are more efficient in vitro.

Important differences in the clinical responses also occur. Although most of the models are lymphopenic, neutropenia is consistently present in South American HF analogues, perhaps related to the bone marrow involvement so prominent in these diseases. The marked thrombocytopenia associated with the South American diseases and their models may have a similar origin and may in turn be responsible for their more extenisve hemorrhagic manifestations. The reason for the higher prevalence of neurological manifestations in humans with South American HFs when compared with Lassa fever is not known, but CNS invasion is the rule in the model Junin and Machupo infections studied here. Furthermore, neurological disease in infected guinea pigs or monkeys seems to be temporally related to serum antibody appearance or to its administration.

Certain lesions observed in the models have not been recorded in humans, but demonstrate an important potential of these viruses which should not be overlooked. For example, necrosis of sympathetic ganglia is seen in Junin- and Machupo-infected macaques; similar involvement, if present in humans, could well be responsible for the vascular instability seen during acute illness and which persists into convalescence. Macaques with South American HFs also have extensive epithelial involvement in the nasopharynx and gastrointestinal tract, a process which might occur to a lesser extent in the human gastrointestinal

tract and be responsible for the frequent bleeding observed from that site. A Lassa-infected squirrel monkey with extensive arteritis has been described (WALKER et al. 1975) and occasional large-vessel lesions are seen when large numbers of Lassa virus-infected macaques are available for examination. It seems likely that similar lesions could occur in humans under appropriate circumstances.

There is evidence that the importance of virus-specific factors extends beyond the contrast of Junin and Machupo to Lassa viruses. These models have allowed us to see the variations in tropism and virulence that exist among isolates of the same virus obtained from different sick humans. In the case of Junin virus, the clinical patterns from donor patients correlated with the disease reproduced in rhesus macaques.

Arenaviruses are probably transmitted from rodents to man largely by aerosols, and considerable laboratory evidence exists to support the aerosol infectivity and stability of the viruses in aerosol suspension. These facts must be taken into consideration when recommendations for patient care are formulated.

3 Physiological Studies

Regardless of the virological and immunological events occurring in the course of infectious diseases, the actual impact on the host is most immediately measured through the resulting physiological alterations. This is particularly important in the arenaviral hemorrhagic fevers, since the causative viruses are largely noncytopathic and there is relatively little histopathological evidence of damage to significant target organs. We have chosen the Pichinde-infected, strain 13 guinea pig (Sect. 2.4.2) for initial studies because of the similarity of virological, immunological, and pathological events to those in Lassa fever, and because of the low biohazard of the virus for man (LIU et al. 1982, 1983, 1984a, b).

Adult (300–500 g) animals were inoculated with 10^4 PFU of adapted Pichinde virus and observed in separate cages to allow individual measurements and to minimize stress. The first obvious signs of disease were evident around day 7, when food and water intake decreased and weight loss began. Clinical signs included hyperventilation, increased salivation, decreased activity, and conjunctival injection. Most infected guinea pigs died between days 13–19 (mean day 14), but 7% survived. This contrasted to guinea pigs housed in large pans in groups of 5, where mortality was 100% and death occurred by day 14. Fever was detected as early as day 4 and increased to about 40°C by day 9, with hypothermia as a terminal event. There was a drastic decrease in body weight, falling 25%–30% below preinoculation levels. Water and food intake of infected animals fell 60%–75%; however, uninfected, pair-fed controls lost only 6.5% of their weight during the same period and uninfected guinea fed ad libitum gained 8%. By day 7–10, infected guinea pigs were in negative balance for water, electrolytes, nitrogen, and energy; these disturbances were progressive. Total O_2 or energy consumption was only slightly increased in the infected animals compared with uninfected controls. The mechanism underlying the severe catabolic reaction was not elucidated, but may well have been due to interleukin 1, cachectin (BEUTLER and CERAMI 1986), or other hormones (BEISEL 1983).

3.1 Role of the Heart

Cardiac output was determined by dye dilution studies with indocyanine green in intact, unanesthetized, and restrained guinea pigs cannulated the previous day. A decrease was noted by day 7–10, with further decline to 45% of normal by day 14. Blood pressure was maintained by commensurate increases in peripheral vascular resistance. Preload was not measured directly, but blood volume and plasma volume (Evans blue dye) were normal relative to body weight. Moreover, attempts to improve cardiac performance by fluid loading were not successful (see Sect. 3.5). This suggested that the major problem might lie in the state of the myocardium itself. Intensive examination of the heart by conventional histopathological means failed to detect lesions, and exhaustive attempts to localize viral replication by fluorescent antibody techniques failed. Therefore, a method was devised to study the total working isolated heart of the guinea pig (Liu 1986). With this in vitro heart preparation, a progressive deterioration in myocardial function was found during the course of Pichinde infection. Virtually all pressure and flow parameters studied were affected, including, particularly, cardiac contractility (dp/dt) of the left ventricle. Right ventricular function was abnormal but less affected. This loss of cardiac functional capacity was reflected in changes of composition of the heart. Heart weight actually declined 11% compared to that predicted from control animals of the same body mass as at the beginning of the experiment. The loss was particularly important in the protein content with total heart nitrogen only 59% of the predicted value. There seemed to be little effect on the conduction system, judging from the minor changes noted in the electrocardiogram.

3.2 Renal-Electrolyte

Metabolic acidosis gradually developed, reaching a blood pH of 7.29 by day 14 (Table 9). Lactate and pyruvate rose 50%–100%, but the lactate/pyruvate ratio declined. Furthermore, there was no anion gap. The suspicion that this was in large part a renal tubular acidosis was confirmed when urine pH was found to be alkaline. The presence of a tubular lesion was also supported by the decrease in the Tm for glucose and para-aminohippuric acid (PAH) (Table 10). Glomerular function is decreased in proportion to the fall in cardiac output; renal blood flow (PAH cleareance) and glomerular filtration (inulin clearance) are approximately halved (Liu 1984b). These findings are in accord with virological results; no antigen is present in the glomeruli, and patchy involvement of tubules is seen. There are no significant histopathological lesions.

There is also a disturbance of Na^+ and K^+ metabolism. Serum concentrations are decreased (Table 9), but daily urinary excretion exceeds intake with typical concentrations of 160 mEq/liter Na^+ and 90 mEq/liter K^+ in a urine volume of 115 ml/kg per 24 h. In spite of hypo-osmolar serum, urine osmolality typically exceeds 1000 mosmol/liter. This pattern suggests antidiuretic hormone secretion either autonomously or as a result of functional hypovolemia. Similar observations have been made in AHF (Davalos et al. 1977; Maglio et al.

Table 9. Plasma and blood concentrations in Pichinde-infected strain 13 guinea pigs

	Days postinoculation		
	0($n=4$)	7($n=5$)	14($n=5$)
Plasma			
Na^+ (mEq/liter)	138±2	132±1	128±3*
K^+ (mEq/liter)	5.1±0.3	4.3±0.6	3.2±0.2*
CL^- (mEq/liter)	104±2	99±3	100±3
HCO_3^- (mEq/liter)	29.9±0.4	22.4±1.1*	15.1±2.2**
Glucose (mg/100 ml)	149±6	153±9	221±34*
Urea nitrogen (mg/100 ml)	15±2	14±1	27±4
Blood			
pCO_2 (mm Hg)	39±2	36±2	28±2*
pH	7.38±0.02	7.39±0.01	7.29±0.04

*$P<0.05$ compared to day 0
**$P<0.01$ compared to day 0

Table 10. Renal function in strain 13 guinea pigs infected with Pichinde virus

Parameter	Days postinoculation		
	0($n=6$)	7($n=5$)	14($n=5$)
C inulin (ml/min per kg)	3.5±0.2	2.2±0.1*	1.6±0.1*
Tm glucose (mg/min per kg)	8.5±1.3	4.9±0.3*	3.6±0.5*
Tm PAH (mg/min per kg)	6.0±0.7	1.2±0.1*	1.1±0.1*
C PAH (ml/min per kg)	3.6±3.0	18.0±2.0*	19.0±2.0*

*$P<0.05$

1973). The potential importance of these electrolyte disturbances is obvious. For example, in the Pichinde-infected guinea pig, gavage with 90 ml/kg per 24 h of an alkaline electrolyte solution (Na^+, 54 mEq/liter; K^+, 99 mEq/liter; $C1^-$, 99 mEq/liter; $HCO3^-$, 54 mEq/liter; pH = 8.2) improved average survival from 16 days in sham-treated controls to 20 days ($P<0.01$).

3.3 Adrenal Function

Adrenal infection is a consistent finding in arenaviral HF models; for example in the guinea pig Pichinde virus extensively infects both cortex and medulla. Although there are only minor histological lesions in the gland itself, autonomic ganglia undergo extensive degeneration in experimental primate models of AHF, BHF, and Lassa fever. In our studies of the Pichinde-infected guinea pig, the adrenal cortex actually increased in mass by 80%. Although significant numbers

of these cells were infected, serum cortisol levels rose 12-fold. We interpreted this as an appropriate response to the stress of a lethal viral infection. Aldosterone levels also rose twofold.

In contrast, the weight of the adrenal medulla fell 25% during the first 2 weeks of infection. Although we have no direct measurements of autonomic function or hormone secretion, it may be relevant that infected guinea pigs subjected to a 75° head-up tilt usually die within a few minutes.

3.4 Other Organs

Lung and liver are two major sites of viral replication. Direct measurements of organ water content in the Pichinde-infected guinea pig indicate pulmonary edema, but gas exchange is maintained until the last stages of infection, when severe hypoxia occurs. Hepatic function is well preserved, as measured by serum albumin concentration and clearance of indocyanine dye. In the terminal stages indocyanine green clearance decreases markedly. Reticuloendothelial function is also preserved until the last stages of disease, when carbon clearance falls dramatically.

3.5 Capillary Permeability

No detailed studies of this critical feature of viral HF have been carried out. The presence of pulmonary edema in the wasted guinea pig dying with Pichinde infection is very suggestive, although there are no simultaneous measurements of left atrial pressure to definitively establish that the edema is not cardiogenic. Disappearance of Evans blue dye, which is bound to serum albumin, is normal, suggesting normal permeability to colloid. Changes in cardiac output following fluid challenge are instructive (Fig. 2). Normal guinea pigs respond to human serum albumin infusions with an increase in cardiac output consonant with an increase in preload. Infusion of Ringer's lactate produces a small increase in cardiac output and is well tolerated. Infected animals receiving Ringer's lactate showed transient improvement in cardiac output, but subsequently died with pulmonary edema; however, colloid does improve their cardiac performance. This suggests that electrolytes and fluid are lost from the circulation, while the infused albumin is retained and results in a more favorable position on the Starling curve. Increased lung weight and water content confirm the massive pulmonary edema that accompanies fluid infusion in the infected animal.

3.6 Leukotrienes

The findings of extensive cardiovasular derangement without direct viral involvement of the myocardium led us to conceptualize the problem in terms of the analogous situation of soluble mediators participating in shock syn-

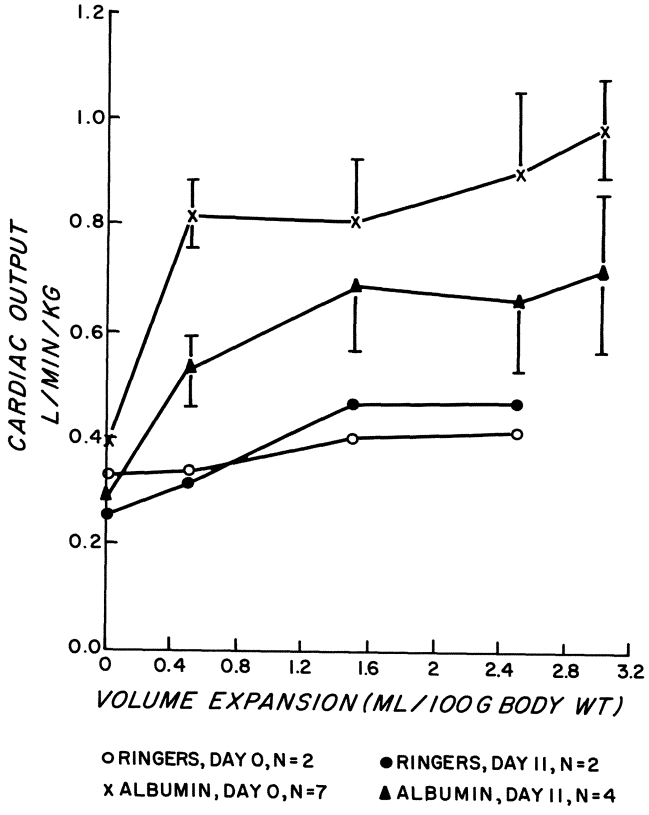

Fig. 2. Change in cardiac output following constant infusion of Ringer's lactate or 25% albumin solution in Pichinde virus-infected strain 13 guinea pigs

dromes. We performed exploratory experiments with several inhibitors, using survival time as a crude index to indicate areas requiring further study. The leukotriene antagonist FPL-55712 prolonged average survival from 14 days to 21 days (Table 11; Lui et al. 1986). Treated animals lost less weight and consumed more food and water. Aspirin (50–100 mg/kg per day, cyclo-oxygenase inhibitor) and naloxone (5 mg/kg per day, endorphin antagonist) resulted in smaller but still significant increases in survival from 14 days to 17 days. Captopril (1.0 mg/kg per day, angiotensin convertase inhibitor) and spironolactone (1.0 mg/kg per day, aldosterone antagonist) had no effect on course of the disease.

Because of the striking effects of FPL-55712 on survival, we measured leukotrienes (LT) in plasma from infected guinea pigs. Radioimmunoassay for LTC_4 was positive, although this test has significant cross-reactivity with other sulfidopeptide leukotrienes, such as the relatively stable metabolite LTE_4. Functional assay on guinea pig ileum showed activity commensurate with radioimmunoassay values. Preliminary studies of FPL-55712 given via constant i.v. infusion have also shown efficacy in acutely reversing the decrement in cardiac output found in the day 10, Pichinde-infected guinea pig.

Table 11. Prolongation of survival of strain 13 guinea pigs treated with the leukotriene antagonist FPL-55712[a]

Group	*n*	Mean time to death (days)
Sham-injected controls	18	14.2±0.5
FPL-55712 (4 mg/kg per day)	5	20.6±1.3*
FPL-55712 (10 mg/kg per day)	3	19.0±1.7*

[a] Drug injected s.c. three times daily. We thank Fisons Pharmaceuticals, Leicestershire, England, for furnishing this compound
* $P<0.05$ compared to controls

Clearly, further study of leukotriene participation in the pathophysiology of arenaviral and other HF is required. They could be solely or partially responsible for several observed pathophysiological processes, particularly decreased myocardial contractility and increased capillary permeability.

4 Issues in Pathogenesis

4.1 Host and Viral Determinants of Pathogenicity

4.1.1 Virus Strain Contrasts

The pathogenetic spectrum of arenaviruses can be understood only when related to host species, genotype, and physiological status (e.g., age, sex, nutrition, immunocompetence, etc.), but comparison of different virus strains is nevertheless instructive. For example, 35 consecutive Lassa virus isolates from patients interned in a single hospital in rural Liberia were tested for their virulence in strain 2 and strain 13 guinea pigs (JAHRLING et al. 1985a). Only two of these isolates were lethal for both strain 2 and 13 guinea pigs and these two viruses were obtained from fatally infected patients. Isolates from severely ill patients (Table 12) were usually lethal for strain 13 but not strain 2 animals (6/7), and viruses from less severely affected patients usually did not kill guinea pigs of either inbred strain (9/14). Isolates from infants and pregnant women were few in number and did not show the same correlation between the severity of human disease and guinea pig virulence. Indeed, most guinea pig-benign isolates obtained from patients with severe or fatal disease were from pregnant women or infants. Although such isolates did not kill adult strain 13 guinea pigs, they were uniformly lethal for 3- to 5-day-old guinea pigs and for pregnant guinea pigs which invariably aborted infected fetuses and died soon thereafter.

Although there are no consistent serological differences among these virus strains, viremia patterns for the inoculated guinea pigs predict the outcome. In lethal infections viremias evolved more rapidly, reached higher titers, and were more sustained than in nonlethal infections (JAHRLING et al. 1985a).

Table 12. Comparison of Lassa fever severity and virulence of patient's virus isolate for guinea pigs

Patient category	Disease severity[a]	Number of Lassa virus isolates lethal for strain 2 or 13 guinea pigs		
		Both 2 and 13	13 only	Neither
Adult	Death	2	1	1
	Severe	0	6	1
	Mild	0	5	9
Pregnant	Death/severe	0	2	2
	Mild	0	1	1
Infant	Death/severe	0	2	2

[a] Patients were classified without knowledge of guinea pig results by these criteria: *severe,* >10 days in hospital, fever >103° F (39.45°C), shock, and/or hemorrhagic signs; *mild,* <10 days in hospital, fever <103°F (39.45°C), no signs of shock or hemorrhage

Different Junin virus isolates also induced characteristic patterns of infection in guinea pigs (Sect. 2.6.1) or rhesus monkeys (Sect. 2.6.2). Although only single isolates were tested, viruses from patients with disease courses characterized as neurologic, mixed, or common produced strikingly similar clinical patterns in macaques. In the guinea pig system several plaque clones of each isolate were examined, with results similar to the parental virus pool, suggesting a viral genetic basis for differing disease patterns. Florid hemorrhagic disease was associated with high viremia and extensive lymphoid and bone-marrow necrosis. Neurological disease occurred later and was associated with lesser viremia but high-titered virus replication in the brain.

Naturally occurring and derived strains of Junin virus not only vary in their disease pattern for primates or guinea pigs, but also seem to have systematic differences in their overall virulence for several host species (CONTIGIANI and SABBATINI 1977). The basis for the covariance of pathogenicity of Junin strains for humans, monkeys, guinea pigs, and infant mice is not understood but has been a valuable tool for vaccine development (Sect. 6.3). In the guinea pig, attenuated strains do not reach high titers and do not grow well in such target tissues as bone marrow and macrophages. Their interactions with the complement (C) system may also contribute to their attenuated phenotype (see Sect. 4.4.3).

Pichinde virus, ordinarily benign for guinea pigs, provides further examples of the spectrum of viral properties that may be readily induced by laboratory manipulation. Serial spleen-to-spleen blind passage or harvest of spleen from the occasional strain 13 guinea pig dying after inoculation of unadapted virus will yield a guinea pig lethal variant. Similar viral types exist in unselected Pichinde mouse-brain pools, since 1 plaque clone in 20 was virulent for guinea pigs. It is important to note that guinea pig-adapted Pichinde does not kill rhesus monkeys or other hosts such as adult mice or outbred hamsters, nor

Table 13. Rapid development of resistance in strain 13 guinea pigs to virulent Lassa virus challenge following inoculation of a Lassa strain benign for guinea pigs[a]

Attenuated virus inoculation		Results of challenge	
Z-158 dose (log_{10} PFU)	Days before challenge	Z-132 dose (log_{10} PFU)	Dead/total
6	0	1	5/5
	1	1	0/5
	2	1	0/5
2	1	1	2/5
	2	1	0/5
6	2	4	1/5
2	2	4	4/5
None	–	1	5/5
		4	5/5

[a] Guinea pigs were inoculated s.c. on day 0 with attenuated Lassa strain Z-158 at a high dose (6 log_{10} PFU) or low dose (2 log_{10} PFU); they were then challenged immediately (day 0) or on days 1 or 2 with virulent Lassa stain Z-132 at either of two doses (1 or 4 log_{10} PFU) s.c.

have attempts to adapt Pichinde virus to produce a murine HF model by sequential spleen-to-spleen passage been successful (JAHRLING, unpublished results).

Since adapted Pichinde (like guinea pig-lethal Lassa virus strains) replicates earlier and to higher titer in strain 13 guinea pigs than its parent (JAHRLING et al. 1981), we compared selected biological properties that have correlated with the virulence of other viruses. Adsorption rates for parental and adapted Pichinde to guinea pig peritoneal macrophages were very similar, as were growth rates in these cells. The rates at which these viruses were cleared from the circulation after i.v. inoculation were also similar (JAHRLING, unpublished observations). Thus, the differences in viremia did not relate to differences in clearance rates or interactions with macrophages.

Interference mechanisms may also regulate the spread of arenaviruses in vivo and in vitro and be important determinants of attenuation. These mechanisms are not well understood, but two observations suggest their existence. First, when supernatant fluids from guinea pig kidney or Vero cell cultures infected with attenuated Lassa viral strains are titrated on Vero cells, plaque formation is inhibited in lower dilutions (e.g., 1:10–1:1000), but not in higher dilutions. Liberian Lassa viral strains of lower virulence tend of form "bull's-eye" plaques, which could also be the result of in vitro interference. Although qualitatively similar interference is also demonstrated with virulent Lassa viral strains, it is only present in undiluted concentrations of infective supernatants. Josiah strain Lassa virus is susceptible to interference, however, since Mopeia supernatant fluids diluted 1:1000 totally inhibited plaque formation in Vero cells (JAHRLING, unpublished data).

The second observation is that attenuated Lassa viral strains can inhibit the replication of virulent Lassa virus in susceptible guinea pigs (Table 13). Infection of guinea pigs with guinea pig-benign virus Z-158 protected against challenge with virulent strain Z-132 as early as 1 day later. The timing of resistance depended on the dose of both the benign and virulent challenge virus strains. In a similar experiment with Mopeia virus given initially, complete protection was afforded against Josiah strain challenge within 12 h; furthermore, challenge viral replication was totally inhibited in target tissues (spleen, lung, adrenal) sampled 7 days after viral challenge. Similar, but less dramatic, early interference has been observed in our laboratory for the attenuated XJ45 and virulent Romero strains of Junin virus in guinea pigs (KENYON, unpublished observations).

4.1.2 Host Determinants

In several cases large differences in susceptibility appear to be directly related to host genetic differences. The exquisite sensitivity of the inbred MHA hamster to Pichinde infection is believed to relate to an impaired delayed hypersensitivity response, and to a relatively high proportion of NK cells in the spleen which serve as a substrate for viral replication (GEE et al. 1979). The delayed hypersensitivity response has been correlated with resistance to LCMV WE infection of inbred hamsters also (GENOVESI et al., unpublished).

Analogous mechanisms may explain the increased susceptibility of strain 13 guinea pigs in comparison with strain 2 animals to Lassa and Pichinde viruses. Higher concentrations of infectious virus are found in target tissues and blood of strain 13 compared with strain 2 animals at all times after inoculation. Furthermore, an effective immune response curtails virus spread and leads to recovery of strain 2 animals. In contrast to the uniform susceptibility of strain 13 guinea pigs to viruses such as Lassa Josiah or adapted Pichinde, 20%–30% of outbred animals survive over a $5 \log_{10}$ range of viral inocula. This may reflect genetic heterogeneity in the resistance of outbred guinea pigs. The critical determinant of survival may be the immune response. The viremias in all outbred guinea pigs are similar to those of fatally infected strain 13 animals until 7–8 days after infection; then, in animals destined to survive, the viremia begins to subside, while in lethally infected animals, the viremia continues to increase (JAHRLING et al. 1982). A similar pattern was observed in rhesus monkeys infected with Lassa (JAHRLING et al. 1980).

There was no difference in the IFA response in guinea pigs or monkeys regardless of outcome. Closer analysis of these models (Sect. 4.2) suggest that N antibody is not responsible for recovery either, but rather that the cellular immune response is the major mechanism. The well-established differences in *Ir* genes among guinea pig strains (BENACERRAF et al. 1967) may be a critical factor in the outcome of these infections. Whatever determines these differences in Lassa and Pichinde infections, outbred guinea pigs are uniformly susceptible to LCMV WE and virulent Junin virus strains.

4.2 Mechanisms of Recovery and Protection

Recovery from and protection against acute arenaviral infection are mediated both by cellular and humoral immune responses. In the case of Old World arenaviruses, cellular mechanisms dominate, but there is a greater potential role for antibody participation with the South American HF viruses.

For example, in the Lassa virus-infected monkey antibodies reacting with acetone-fixed, virus-infected cells (IFA; Fig. 1) or virus-specific antigens in cell culture supernatants (IgM capture ELISA; Fig. 3) are detected by day 10 of infection. Nevertheless, there is no concomitant decrease in viremia, nor is there any difference in the timing or magnitude of this response between fatal and nonfatal infections. However, the circulating viral antigen detected by an antigen capture ELISA declines coincident with the appearance of virus-specific IgM antibodies (see Sect. 4.4.1). Early convalescent sera from these monkeys react with both NP and GP2 virion polypeptides by Western blot analysis (JAHRLING and BUCHMEIER, unpublished observations). It is possible to show reactivity to GP1, but bands are diffuse and occur only with late-convalescent or hyperimmune Lassa sera. Furthermore, in the case of related LCMV, the critical neutralizing epitope is conformation-dependent and does not react in Western blot tests (BUCHMEIER et al. 1986). Regardless of the biochemical reactivity of conva-

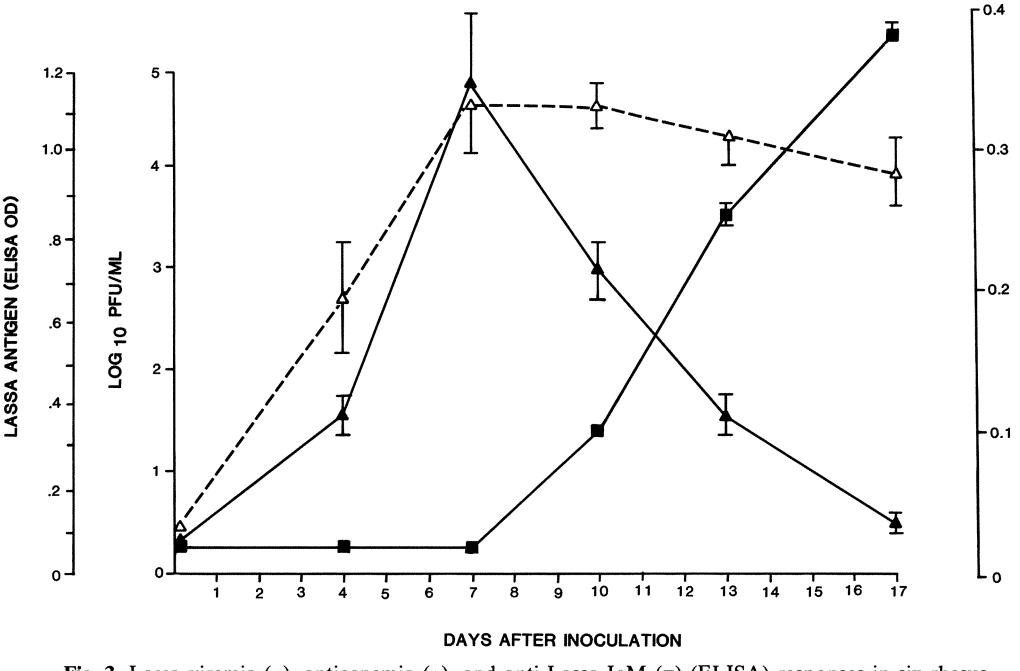

Fig. 3. Lassa viremia (△), antigenemia (▲), and anti-Lassa IgM (■) (ELISA) responses in six rhesus monkeys lethally infected with Lassa virus (Josiah strain). Antigen was determined by a capture ELISA using polyclonal guinea pig and monkey sera. The IgM antibody test was an IgM capture ELISA with anti-IgM bound to plates followed by test serum, viral antigen, anti-Lassa IgG, and labeled anti-IgG (adapted from NIKLASSON et al. 1984)

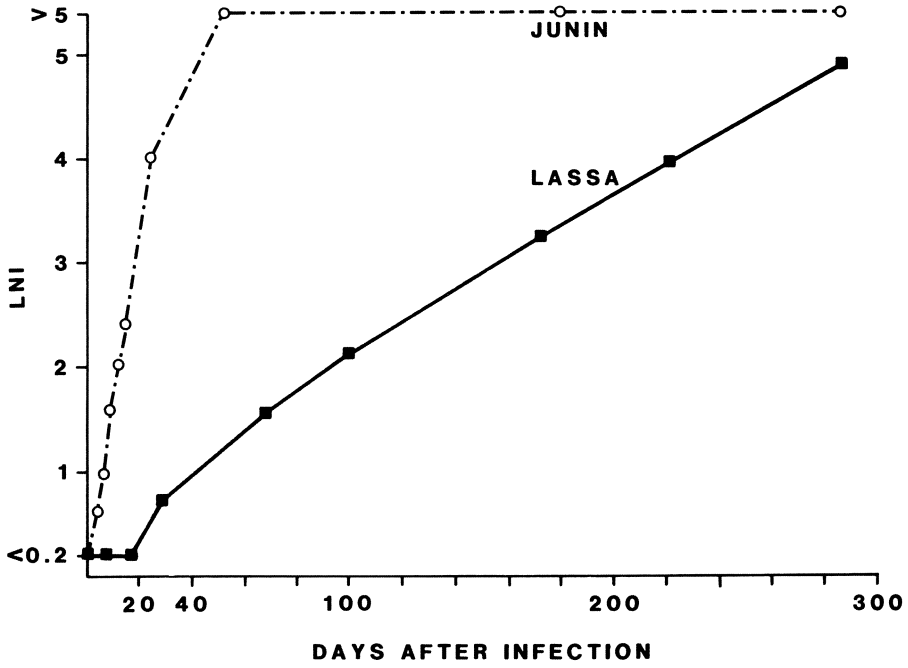

Fig. 4. Evolution of neutralizing antibodies after Junin (XJ clone 3) or Lassa (Josiah strain) infection in surviving rhesus monkeys. Titers are expressed as log neutralization indexes (LNI), as detailed in legend to Fig. 1

lescent antibodies, their capacity to mediate in vitro virus neutralization does not develop until several weeks after cessation of viremia (Fig. 1). In marked contrast, monkeys develop a more rapid neutralizing antibody response after Junin infection (Fig. 4). The late appearance and unusual characteristics of the Lassa neutralizing antibody response, discussed in detail in Sect. 5.2, argue against its playing a significant role in recovery.

Comparison of in vitro measures of the immune response and syngeneic cell transfer in Lassa and Junin infections of guinea pigs extend these observations. Strain 13 guinea pigs infected with the attenuated XJ44 strain develop antibodies detectable by lysis of labeled target cells in the presence of complement, immunofluorescence of acetone-fixed infected cells, and neutralization of virus (Fig. 5). Cytotoxic antibodies are found several days before the virus-neutralizing response. Lysis of syngeneic virus-infected target cells by spleen cell suspensions can be detected somewhat sooner, as early as day 6 (KENYON and PETERS 1986). Lytic activity of effector spleen cells was shown to be due to antibody-dependent cellular cytotoxicity (ADCC). Killing was blocked by aggregated human gamma globulin, remained after treatment with a T-cell specific monoclonal antibody plus complement, and was diminished by maneuvers that enriched spleen cell suspensions for T-lymphocytes. When we attempted to reconstruct the system with normal guinea pig spleen cells and exogenous antibody, we encountered only minimal cytotoxicity. This seeming inconsistency

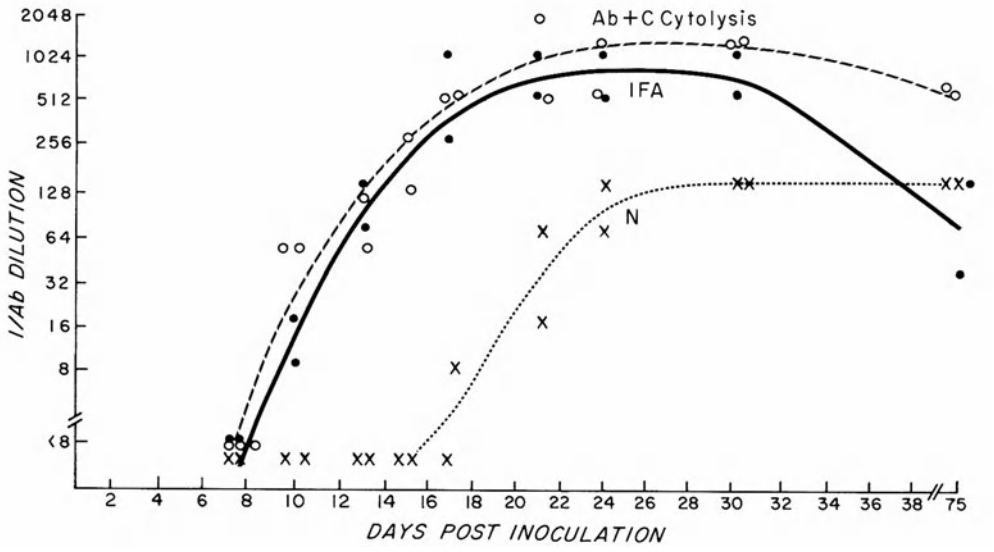

Fig. 5. Humoral immune response of outbred guinea pigs infected with the attenuated XJ44 strain of Junin virus. *N*, neutralizing antibody measured by reduction of 80% of viral plaques; *IFA*, indirect fluorescent antibodies measured on acetone-fixed, Junin virus-infected Vero cells; *Ab+C cytolysis*, lysis of Junin-infected, ^{51}Cr-labeled Vero cells in the presence of guinea pig complement

was resolved when we found that the ability of XJ44-infected guinea pig spleen cells to function as effectors in a nonviral system was greatly increased over that of normal splenic lymphocytes. Although we could not detect antiviral antibodies in supernatants from spleen cell cultures by sensitive ELISA techniques, the very low antibody concentrations capable of mediating ADCC are well known (PETERS and THEOFILOPOULOS 1977).

Adult guinea pigs infected with a virulent Junin strain (Tables 7 and 8) or immunosuppressed with CY and given an attenuated virus such as XJ44 will die with little or no detectable serum antibody. In both cases spleen cells efficiently mediate ADCC against nonviral targets. In contrast, the ontogeny of resistance to Junin virus parallels the development of virus-specific ADCC as well as the ability of spleen cells to lyse nonviral target cells in the presence of antibody (KENYON and PETERS 1986; KENYON et al. 1985).

While virus neutralization is obviously of potential benefit in Junin infections, the importance of the eradication of infected cells is supported by several arguments: (1) early detection of cytolytic mechanisms in vitro following nonlethal Junin infection, (2) the parallel between effective ADCC and resistance, and (3) the rapid clinical improvement and decrease in interferonemia after convalescent plasma therapy in humans (Sect. 4.5).

The role of cellular immunity directed against virus-infected cells is dramatic in the Lassa guinea pig model. Passive transfer of spleen cells, but not plasma, from animals recently recovered from Lassa virus infection protects recipients against lethal challenge (Table 6). These spleen cells are actively cytotoxic for Lassa-infected syngeneic target cells (Table 5). Furthermore, guinea pigs infected

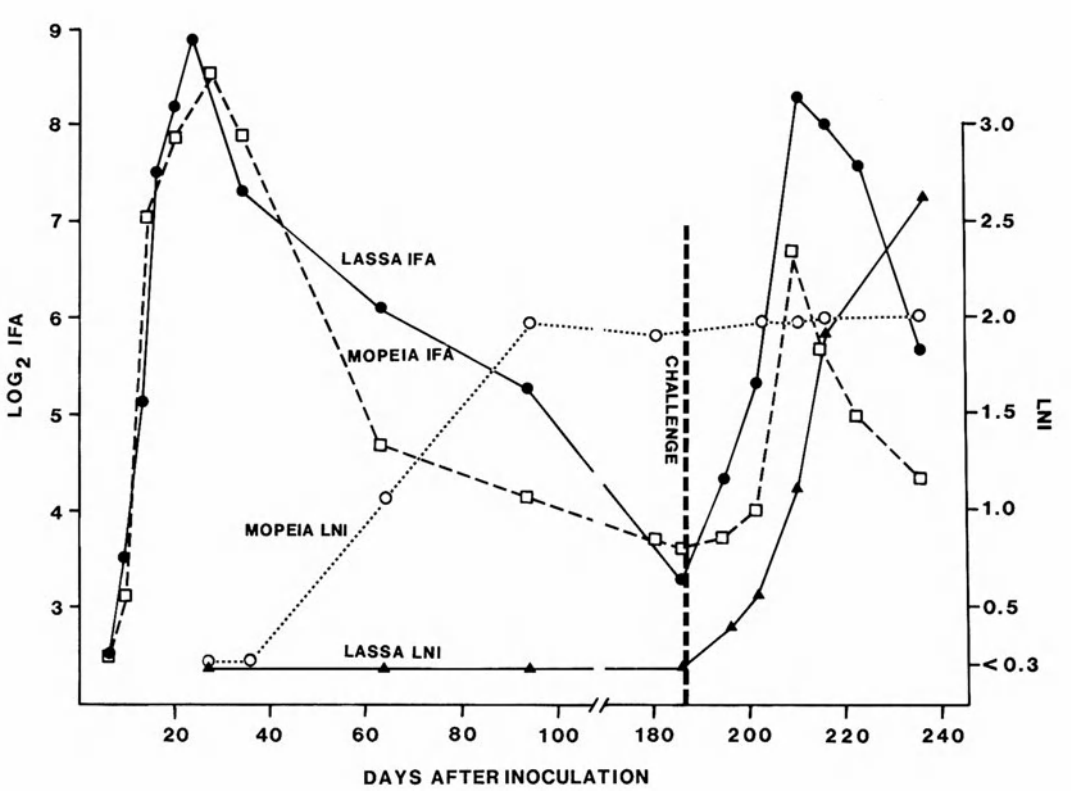

Fig. 6. Antibody responses to Lassa and Mopeia viruses in six rhesus monkeys inoculated s.c. on day 0 with 4.1 $\log_{10}$ PFU Mopeia virus and challenged s.c. on day 187 with 4.6 $\log_{10}$ PFU Lassa virus (strain Josiah). Immunofluorescent antibody (*IFA*) titers were determined with acetone-fixed Vero cells infected with Lassa or Mopeia virus and a conjugate against monkey total immunoglobulins. Neutralizing antibody concentrations were quantitated as $\log_{10}$ neutralization indexes (*LNI*) against Mopeia or Lassa (Josiah) virus (see Fig. 1 legend)

with the lethal Josiah strain of Lassa virus do not develop cytotoxic spleen cells, perhaps another expression of the immunosuppression observed in the arenaviral HF (Sect. 4.2).

Studies of cross-protection between different arenaviruses also provide useful information on host defense mechanisms which can control infection. For example, monkeys immunized with Mopeia virus developed a modest LNI of 2.0 to Mopeia virus, but no detectable neutralizing antibody to Lassa virus (Fig. 6). When challenged with Lassa, no clinical disease or viremia was detected, but the Lassa LNI rose in an accelerated fashion (contrast Figs. 1 and 6). This anamnestic neutralizing antibody response was too late to be responsible for suppression of Lassa viremia. There was no change in the LNI to Mopeia virus, but the IFA to both Lassa and Mopeia rose 8- to 32-fold. Interestingly, in contrast to primary Mopeia or Lassa infections, no antibodies were detectable with IgM-specific fluorescein conjugates, perhaps due to IgG competition.

Once again, it was possible to analyze the mechanism of Old World arenavirus cross-protection in more detail using the guinea pig model (Sect. 2.3.1). Protection often occurred in the absence of cross-reactive neutralizing antibodies (Table 4), was adoptively transferred with spleen cells (Table 6), and followed the pattern of cross-reactivity seen in cell-mediated cytotoxicity assays (Table 5).

Tacaribe virus also immunized guinea pigs and marmosets against Junin virus challenge (TAURASO and SHELOKOV 1965; COTO et al. 1967, 1980; WEISSENBACHER et al. 1982). Tacaribe-immunized animals developed neutralizing antibody to Tacaribe but not to Junin virus, yet they resisted challenge and developed an anamnestic neutralizing antibody response to Junin. In another example, rhesus monkeys immunized with attenuated Junin XJ clone 3 developed high titers of neutralizing antibody to Junin virus, but negligible titers to Machupo, yet they resisted Machupo virsu challenge and developed an accelerated neutralizing antibody response to the challenge virus (EDDY and JAHRLING, unpublished observations). It is not clear in either case whether protection was due to cellular immunity or cross-priming for an accelerated antiviral humoral response.

We have not touched on nonimmunological mechanisms which might contribute to recovery from acute infections. For example, murine macrophages infected with LCMV in vitro decrease their infectious virus output over a period of several days in culture and simultaneously acquire the ability to activate spontaneously the alternate C pathway (WELSH and OLDSTONE 1977). Both phenomena would tend to limit viral spread and enhance the sensitivity of infected cells to lysis. The direct antiviral effects of interferon (IFN) probably do not play an important role in arenaviral infections, since high concentrations of IFN show little such activity in cell culture systems (see Sect. 4.5).

Thus, the dominant immunological mechanism responsible for recovery and protection in Old World arenavirus infections seems to be the cellular immune response, presumably T-cell mediated. Similar mechanisms are undoubtedly operative in Junin and Machupo infections, but the early antibody response is more efficient in virus neutralization and in adoptive transfer of protection. This antibody may complement or overshadow the role of cellular immunity.

4.3 Immunosuppressive Effects of Infection

The frequency of pyogenic secondary infections (BUSTOS et al. 1975; PETERS et al. 1974) in South American HF suggests that polymorphonuclear (PMN) leukocyte function is compromised. This is also seen in Junin-infected monkeys which have purulent conjunctivitis and other intercurrent bacterial infections (MCKEE et al. 1985b). Leukocyte dysfunction may be attributable to direct interactions of Junin virus with PMN cells (LAGUENS et al. 1983a), bone marrow necrosis, maturation arrest, and leukopenia (CARBALLAL et al. 1977). Studies of classical immune function in the guinea pig model of AHF demonstrated necrosis of macrophages, T- and B-lymphocyte depletion, decreased primary and secondary antibody responses, blunted Arthus's reaction, and anergy after established tuberculin sensitivity (FRIGERIO 1977; CARBALLAL et al. 1981). Hu-

mans also have a profound decrease in recall of delayed hypersensitivity, mitogen responsiveness of peripheral blood leukocytes, circulating levels of B cells and T cells, with an inversion of the T4/T8 ratio (ARANA et al. 1977; ENRIA et al. 1986b). IFN (Sect. 4.5) and defective macrophage function are high on the list of candidate causes for these defects. Virulent Junin strains infect and damage macrophages extensively in vivo (Sect. 2.6.1; GONZALEZ et al. 1980). Indeed, attenuated Junin strains grow to lower titers in guinea pig lymphoid tissues and are not immunosuppressive (GALASSI et al. 1982). Furthermore, when dendritic cells or macrophages are isolated from infected guinea pig spleens, attenuated strains replicate only in dendritic cells, whereas virulent virus grows in both cell types (LAGUENS et al. 1983b).

Perhaps the most obvious example of the immunosuppressive effects of these viruses is the delayed immune reactivity to the agents themselves, requiring 10–20 days for effective responses. The unusual nature of neutralizing antibodies to Lassa, LCM, and Pichinde viruses is also pertinent (Sect. 5.2.).

4.4 Immunopathogenesis

Classical studies of disease induction in the LCMV-infected mouse focused on the role of host-immune response to viral antigens in actually causing the observed disease. In contrast, there is no direct evidence that the immune response exerts an adverse effect in the arenaviral HF. For example, the guinea pig fatally infected with a virulent Junin virus strain such as Romero never develops detectable virus-specific humoral or cytotoxic spleen cell responses. Immunosuppression with cyclosporin A or CY does not ameliorate disease. The same regimens of these drugs ablate the immune response to attenuated Junin viruses and convert benign to lethal infections (KENYON et al. 1985; KENYON and PETERS 1986). Similar but less extensive results have been reported from Machupo-infected monkeys (EDDY et al. 1975a), Pichinde-infected guinea pigs (E. JOHNSON, unpublished data), Pichinde-infected hamsters (MURPHY et al. 1977), LCMV-infected guinea pigs (BUCHMEIER et al., unpublished results), and LCMV-infected hamsters (GENOVESI et al., unpublished results; see Sect. 2.1.2). In spite of these negative experiments, there sill may be a role for the participation of the immune system in host damage.

4.4.1 Immune Complexes

Immune complexes are established mediators of immunopathology in several disease states, including the chronically viremic LCMV-infected mouse. However, histological studies of man and experimental animals with arenaviral HF have not shown the classical vascular or glomerular lesions of serum sickness or immune complex nephritis. In AHF patients, convincing studies have failed to detect Clq-binding immune complexes, classical C pathway activation (DE BRACCO et al. 1978), or glomerular deposition of Ig, C3, and viral antigens (MAIZTEGUI et al. 1975). Arteritis suggestive of or consistent with immune com-

plex causation has been observed in several nonhuman primates infected with Lassa virus and hamsters infected with LCMV (WALKER et al. 1975; CALLIS et al. 1982; GENOVESI et al., unpublished observation).

Several other observations are very suggestive of immune complex formation in arenaviral HF. In Lassa (man, monkey, guinea pig), LCMV (monkey, hamster), and Pichinde (guinea pig) HF infections, IFA can be detected after the onset of disease and before viremia begins to diminish. The specificity of these antibodies for virion polypeptides has not been explored in depth. For example, in the Lassa-infected monkey (NIKLASSON et al. 1984) and human (JAHRLING et al. 1985c), viral antigen is readily demonstrated by ELISA tests of acute sera. With the appearance of virus-specific IgM antibodies, antigen can no longer be detected in the antigen-capture test used, but in many cases viral infectivity remains in the same or actually increases in titer. When sera from infected monkeys are mixed with anti-IgM antisera, viral infectivity is neutralized (JAHRLING, unpublished observations), although addition of protein A-bearing staphylococci and subsequent centrifugation (JAHRLING et al. 1978) has no effect. Presumably these virus-IgM complexes fail to be effectively removed from the circulation for the same reasons that IgM-coated erythrocytes are only transiently cleared (FRANK et al. 1977). It seems likely that under certain circumstances of host, virus, and immune response, phlogistic immune complexes might participate in the clinical manifestations of VHF, but it appears that they do not do so regularly.

4.4.2 T Cells

T-lymphocytes are, of course, central to the immunopathology of the LCMV-infected adult mouse. Critical studies with passively transferred LCMV-specific T-cell lines have shown that cytoloytic T-lymphocytes, in particular, are sufficient to induce the fatal convulsive disorder (BAENZIGER et al. 1986). Since global immunosuppression does not spare animals from disease, it is open to question whether these effectors or other cells of thymic lineage play a special role in causation of the VHF syndrome. This issue will not be finally resolved until the T-lymphocyte biology of primates or guinea pigs is better understood or a realistic murine VHF model is available.

4.4.3 Complement

There are modest abnormalities of C component levels in human AHF, but no clear pattern of classical or alternate pathway activation (DE BRACCO et al. 1978). These abnormalities may be due to the release of proteases into the circulation, since serum from Junin-infected guinea pigs contains soluble enzymatic activity which degrades C in vitro (RIMOLDI and DE BRACCO 1980).

C itself may play a critical role in recovery from disease and in the attenuated phenotype of some Junin viral strains (KENYON and PETERS, unpublished observations). Exposure to fresh normal serum produces 10- to 100-fold decreases

in infectivity of attenuated (but not virulent) viral strains. This activity appears to proceed by the classical C pathway in that it is blocked in C4-deficient or EGTA-containing serum, but not by heating at 50° C for 20 min, and no inactivation is seen in sera heated at 56° C for 30 min or in C6-genetically deficient serum. This activity, unlike that seen with some strains of LCMV (WELSH 1977) does not depend on antibody to host cell determinants incorporated into the virus or on low levels of naturally occurring antiviral antibodies. C also plays an important role in antibody-mediated virus neutralization. Although no antigenic differences are detected between virulent and attenuated virus strains in conventional plaque-reduction neutralization tests, the presence of C enhances serum titers 10- to 20-fold when tested against virulent strains. Cells infected with virulent viruses in vitro also are less sensitive to antibody and C cytolysis (KENYON and PETERS, unpublished observations).

4.4.4 Antibody-Dependent Enhancement

Nonneutralizing IgG antibody bound to the surface of many different viruses can enhance infection of Fc receptor-bearing cells in vitro and is an important determinant of some cases of dengue HF (PETERS and JOHNSON 1984). There is no evidence that this mechanism is operative in arenaviral HF, even when marginal amounts of IgG are infused into infected animals (Sect. 5.2). However, IgG does have a remarkable effect in the Machupo monkey model (EDDY et al. 1975b). Human IgG was given i.m. 4 h after virus inoculation to simulate prophylaxis of a laboratory exposure to Machupo virus. Low doses of IgG were not protective, and moderate doses prevented illness. Unexpectedly, high doses, while protecting against acute illness, resulted in the late onset of neurological disease. This syndrome occurred much later than the usual neurological phase of the illness associated with viral invasion of the CNS. The unique pathogenesis of this syndrome is attested by the failure of repeated attempts to isolate virus or detect viral antigen in brains from moribund animals (EDDY, unpublished observations) and by the unusual histopathology (MCLEOD et al. 1976).

4.5 Involvement of Soluble Mediators

A common theme in arenaviral HF of humans and animals is the lack of histological lesions to explain disordered organ function and death. Fluorescent antigen tracing has shown that involvement of parenchymal organs is much more extensive than expected, and infection, per se, may be inducing altered function without overt histopathology, a thesis well developed for LCMV (OLDSTONE 1984). Unfortunately, there are few direct data bearing on the application of this concept to the viral hemorrhagic fevers. Other lines of evidence have led to speculation that soluble mediators may be responsible for many of the clinical manifestations of the hemorrhagic fevers. This is particularly true for endothelial cell involvement. Although these viruses readily infect endothelial cells in culture (ANDREWS et al. 1978), in vivo tracing usually shows much more prominent evidence of macrophage involvement.

The most direct evidence comes from the study of human AHF (LEVIS et al. 1984, 1985) in which serum interferon reached levels of 1000–64000 IU/ml. These enormous concentrations of interferon correlate quantitatively with disease manifestations such as fever, chills, and backache. Other prominent clinical findings in AHF are also known effects of interferon toxicity. The antiviral activity in several sera was acid-stable and was neutralized by antisera to α but not β or γ interferons. Unlike the situations in most viral infections, there was no correlation of viremia and interferonemia. High interferon levels were, however, strong predictors of a fatal outcome. Infusion of immune plasma rapidly cleared viremia, reduced interferon levels, and improved the patients' clinical well-being. Thus, Junin virus, relatively insensitive to interferon in vitro, induces very high levels of α interferon in vivo, which appear to be responsible for many of the clinical manifestations of AHF.

The leukotrienes, suggested as important mediators of tissue ischemia and shock, are also important considerations (LEFER 1985). They have been identified in the plasma of Pichinde-infected guinea pigs, and their inhibition prolongs survival (LIU et al. 1986). Circulatory dysfunction, including cardiac depression and increased vasvular permeability, are established effects of the peptide leukotrienes (LTC4, LTD4, LTE4), as well as important manifestations of the VHF syndrome (FEUERSTEIN 1985). These leukotrienes may well be produced by macrophage (SCOTT et al. 1983), perhaps under stimulation by immunological reactions, complement fragments (HARTUNG and HADDING 1983), immune complexes (FERRERI et al. 1986), or other mechanisms.

The prostacyclin-thromboxane A2 (TXA2) system also may play an important role in the VHF syndrome. These two cyclo-oxygenase metabolites of arachidonic acid are highly active in regulating platelet and endothelial cell function in the microcirculation (HIGGS and MONCADA 1983). Studies of Ebola HF (FISHER-HOCH et al. 1983, 1985b) and Lassa fever (FISHER-HOCH et al., unpublished observations) in rhesus macaques have shown that circulating platelets are refractory to collagen or adenosine diphosphate aggregation. Although the ketamine anesthesia used may have interfered with aggregation studies (ATKINSON et al. 1985), platelets were depleted of platelet factor 4, and plasma levels were elevated. These "exhausted" (O'BRIEN 1978) platelets may have arisen from several possible mechanisms, but an attractive hypothesis was based on the lack of prostacyclin production by aortic rings from an Ebola-infected monkey (FISHER-HOCH et al. 1983). Lack of proper endothelial prostacyclin production could have led to local platelet release reactions, thus enhancing microcirculatory compromise.

Circulating proteases have also been suggested as soluble intermediaries, particularly in the pathogenesis of Junin virus infection. Proteolytic activity was detected in the plasma of infected guinea pigs early in disease (KIERSZENBAUM et al. 1970), and the hypocomplementemia of these animals may well have been mediated by a serum factor that has been shown to inactivate C in vitro (RIMOLDI and DEBRACCO 1980). Such a mechanism could explain the presence of normal or elevated circulating levels of C4 and factor VIII when measured immunologically, but with low functional activities (DEBRACCO et al. 1978; MOLINAS and MAIZTEGUI 1981). PMN leukocytes are candidate sources

as has been postulated in granulocytic leukemias (EGBRING et al. 1977). These cells contain neutral proteases capable of cleaving C4 and liberating active chemotactic factors from C3 and C5 (VENGE and OLSSON 1975), as well as degrading factor VIII (KOPEC et al. 1980). Enzymes could be released as a consequence of bone marrow necrosis (Sect. 2.6), direct interaction of Junin virus with PMN (LAGUENS et al. 1983c, 1986), soluble immune complexes, or other mechanisms. Further support for PMN activation comes from the finding of ultrastructural evidence of cytoplasmic dissolution in 40% of circulating PMN within 7 days, and 80% within 11 days, of guinea pigs infected with Junin virus (CARBALLAL et al. 1977).

The accumulating evidence for PMN involvement in Junin, and perhaps other, HF also focuses on the potential of reactive oxygen species to cause tissue damage. This is particularly pertinent to the pulmonary pathology which seems to be a feature of most viral HF and their animal models. Increased pulmonary vascular permeability seems virtually always to result from oxidative injury, often by neutrophil products (HENSON et al. 1982; TAYLOR et al. 1985). For example, the pulmonary edema associated with complement activation or LTB_4 is produced via oxidative damage from PMN (LEWIS and GRANGER 1986).

It is beyond the scope of this review to speculate on the myriads of other candidate soluble mediators such as kinins, platelet-activating factor, etc., on the complex interactions at the biochemical level (e.g., C, clotting, kinin system, and Hageman factor) or cellular level, where complex stimulatory and inhibitory schemes have been developed both with the inflammatory mediators and specific immunological stimuli, and with the state of cell differentiation or activation.

4.6 Genesis of Hemorrhage

Bleeding at epithelial surfaces is a frequently noted feature of arenaviral HFs, particularly BHF and AHF. Studies of Junin virus infection in guinea pigs (MOLINAS et al. 1978), and both Junin (MCKEE et al. 1986) and Machupo (SCOTT et al. 1978) virus infections in rhesus monkeys, have yielded useful insights into these disease processes. Platelet counts are significantly depressed within 7 days following infection. Thrombocytopenia is progressive, with counts reaching their nadir at or near the time of death. Coincident with falling platelet numbers, progressive necrosis of bone marrow occurs, suggesting that thrombocytopenia may result from impaired production or release of platelets at the marrow level (MOLINAS et al. 1978; TERRELL et al. 1973; GREEN et al. 1986). In *Callithrix jacchus* infected with Junin virus, however, progressive thrombocytopenia occurs most often in the absence of histological marrow abnormalities, indicating that in this model peripheral (probably intravascular) destruction may be important (MOLINAS et al. 1983).

Abnormalities in the coagulation cascade are common to all South American HF models studied to date (MOLINAS et al. 1978, 1983; SCOTT et al. 1978; MCKEE et al., unpublished observations). At 1–2 weeks postinfection, progressive prolongation of activated partial thromboplastin time (APTT) occurs, accompanied by mild to moderate depression of factors II, V, and VIII. Other

clotting factors have been affected to variable degrees as well. Fibrinogen is normal or mildly depressed initially, then increases with desease progression. Fibrin monomers are detected in guinea pigs infected with Junin virus, indicating activation of coagulation up to the level of thrombin (MOLINAS et al. 1978). However, fibrin degradation products (FDP) are detected only at low levels (8–30 μg/ml), or not at all. These alterations occur in the setting of hypotension, mild hepatocellular necrosis, and limited activation of the complement system.

In Lassa fever bleeding manifestations are less common, and the degree of thrombocytopenia is correspondingly less. This is reflected in the results of guinea pig (JAHRLING et al. 1982) or monkey (JAHRLING et al. 1980) inoculations. In Lassa-infected macaques there is modest prolongation of the APTT, and FDP are less than 10 μg/ml; fibrinogen turnover and platelet survival are normal (LANGE et al. 1985). In a Pichinde infection of guinea pigs, which resembles Lassa fever in many respects, the pattern is different (COSGRIFF et al. 1986). Prothrombin time is prolonged, while APTT is shortened. Most clotting factors are decreased, including antithrombin III, but fibrinogen levels are markedly increased. FDP levels exceed 100 μg/ml. Platelets fall modestly, and platelet function appears markedly depressed. Thus, disseminated intravascular coagulation may participate in the clotting abnormalities seen in this model infection.

While the sequence and patterns of change in the hemostatic parameters vary somewhat from model to model, taken together they suggest that platelet abnormalities (quantitative, functional, or both), together with alterations of the coagulation system, probably account for the observed effects. There are suggestions that thrombocytopenia caused by increased platelet consumption or decreased platelet production, combines with impaired platelet activity to render platelets functionless (see Sect. 4.5). There is no evidence for circulating coagulation inhibitors; hepatocellular dysfunction leading to depressed factor synthesis, together with an as yet undetermined mechanism for activation of the coagulation cascade, are likely causes of diminished clotting factor activity. Candidates for an intravascular activation mechanism include hypotension with shock, serum proteolytic enzymes, interaction with other activated systems as the complement or kinin pathways, or endothelial abnormalities. With the exception of the Pichinde-guinea pig model, there is little evidence for disseminated intravascular coagulation. No intravascular fibrin deposition of consequence has been observed, FDP are not present at significant levels, and the patterns of coagulation factors and fibrinogen are uncharacteristic.

4.7 Neurological Disease

Neurological manifestations are common in arenaviral disease (OLDSTONE and PETERS 1978; PETERS 1984) and occur in the great majority of patients with South American HF (BIQUARD et al. 1969). Some cases of AHF pursue a course dominated by CNS signs (RUGIERO et al. 1960). The lack of easily identified residua, and the failure to isolate virus from the brains of patients dying from AHF or BHF have led clinicians to regard this serious and even fatal neurological dysfunction as a manifestation of an encephalopathy rather than a true

viral encephalitis. Certainly, hepatic encephalopathy provides a clinical example of major CNS disease that is completely reversible and, in its acute manifestations, due to purely metabolic alterations (SCHENKER and HOYUMPA 1984). The cerebral manifestations of systemic lupus erythematosis provide a paradigm in which structural correlates are minimal and in which such effector mechanisms as circulating immune complexes, inflammatory mediators, and antibodies reactive with endothelium or nervous tissue have been suggested (HARRIS and HUGHES 1985). The neurotoxicity of interferon has also been invoked (LEVIS et al. 1985).

Animal models, particularly nonhuman primates, resemble the picture seen in South American HFs (Sects. 2.5 and 2.6). The incidence of CNS involvement after experimental Junin infection is clearly related to the viral strain used. Neurological disease in the model systems overlaps, but tends to occur somewhat later than the vasculopathy, while both evolve simultaneously in man. Virus antigen is readily demonstrated in the CNS of monkeys or guinea pigs dying acutely from Junin or Machupo infection. Machupo-infected macaques dying later after ribavirin (STEPHEN et al. 1980) or immune plasma therapy (Sect. 4.4.4), however, have no virus or viral antigen detectable in the CNS.

Lassa virus, in contrast, causes less prominent CNS disease in man and is much less neuroinvasive in model systems. Understanding these differences is of scientific interest, and also impacts on the design of supportive and virus-specific treatment strategies.

5 Development of Therapeutic Measures

5.1 Ribavirin and Other Antivirals

Ribavirin is a nucleoside analogue whose antiviral activity is thought to relate to a competitive inhibition of guanosine in the 5′ capping of viral mRNA. It is effective against several RNA and DNA viruses, both in cell culture and animal models. Resistance has not yet been observed. Its major toxicity appears to be a dose-related, reversible anemia, and it causes little or no immunosuppression (CANONICO 1983). Owing to ribavirin's favorable properties, we tested the drug against several exotic virus infections, including arenaviral HF (STEPHEN and JAHRLING 1979; STEPHEN et al. 1980). Replication of Lassa virus was shown to be inhibited by ribavirin concentrations as low as 10 μg/ml in two types of primate cells (Vero cells and primary rhesus alveolar macrophages). Production of infectious virus, viral antigen accumulation in infected cells, and spread to other cells in culture was markedly reduced (JAHRLING et al. 1980).

The initial study in rhesus monkeys demonstrated the efficacy of ribavirin in treatment of Lassa fever (JAHRLING et al. 1980) and provided the experimental basis for proceeding to clinical trials in man (MCCORMICK et al. 1986). Four rhesus monkeys treated initially on the day of viral inoculation (50 mg/kg loading dose followed by three i.m. doses daily of 10 mg/kg) experienced only mild clinical disease; four monkeys treated initially on day 5 experienced a more

severe illness, but survived. Similar data were obtained for cynomolgus monkeys treated with a slightly different regimen (75 mg/kg loading dose followed by 30 mg/kg per 24 h given in divided i.m. doses at 12-h intervals (JAHRLING et al. 1984). Treated monkeys experienced significantly decreased viremias, and all those treated initially on days 0 or 4 survived; however, only four of eight monkeys treated initially on day 7 survived. Effective ribavirin therapy of Lassa-infected macaques was always initiated before viremia exceeded 4.0 $\log_{10}$ PFU/ml and was followed by a further decline. Serum transaminase values did not rise to the levels seen in lethally infected control monkeys. Recovery was complete, although a transient anemia occured.

Attempts to increase survival by using higher doses of ribavirin were not successful; maintenance doses of 60 or 90 mg/kg induced severe anemia in macaques (WANNARKA et al. 1982), which are more sensitive than man to this side-effect of the drug. An alternative strategy utilizing ribavirin plus high-titered, Lassa-immune plasma resulted in dramatic reductions in viremias in all treated monkeys and survival, even in monkeys treated initially on day 10, when they were seriously ill (JAHRLING et al. 1984). The mechanism of this additive or synergistic effect is not known.

Ribavirin is also effective against Machupo and Junin infections in vitro and in the guinea pig and primate models (STEPHEN et al. 1980; KENYON et al. 1986a). In Hartley strain guinea pigs infected with 2.0 $\log_{10}$ guinea pig lethal doses of Malale strain Machupo virus, significant ($P<0.001$) improvement in survival was seen following treatment with ribavirin or ribavirin triacetate (75 mg/kg loading dose, then 50 mg/kg per day from days 0 through 10) in comparison with sham-treated controls. Rhesus macaques infected with 1000 PFU of Machupo, then treated with ribavirin (or its triacetate) in a variety of dose regimens, showed significant ($P<0.001$) depression of viremia and survival of the acute (hemorrhagic) phase of BHF in comparison to placebo-treated controls. Results were similar, regardless of whether the drug was instituted at the time of infection (day 0) or at onset of fever (days 4–7 postinfection). However, all survivors ultimately developed progressive neurological dysfunction and died. Neither virus nor viral antigen was detectable at necropsy.

Administration of ribavirin to rhesus monkeys infected with Junin virus had an effect similar in many respects to that seen in the BHF monkey system. Sham-treated animals infected with 10^4 PFU of Espindola strain developed typical AHF. Monkeys treated with ribavirin on a prophylactic schedule (60 mg/kg per day for 4 days, 30 mg/kg per day for 3–5 days, then 15 mg/kg per day for 11 days) seroconverted but did not develop viremia or clinical AHF. When the drug (60 mg/kg per day for 5 days, then 15 mg/kg per day for 14 days) was begun on day 6 after onset of viremia and clinical signs, viremia ceased within 2 days, and clinical disease resolved over a 2-week period. Within 2 weeks of cessation of therapy a late-onset neurological syndrome, similar to that which occurred in ribavirin-treated BHF monkeys, appeared and was fatal in two of three macaques. The third animal recovered fully.

Hartley strain guinea pigs infected with a uniformly lethal strain of Junin virus (Romero) were treated daily with ribavirin or tributylribavirin (45 or 58 mg/kg, respectively) in quantities predetermined to be close to the maximally tolerable dosage. There was no increase in survival in the treated animals, but

mean time to death was increased from 16 days to 19–26 days ($P<0.0001$) (KENYON et al. 1986a). Animals dying under treatment were usually paralyzed and high titers of virus were present in their brains. Brain virus was sensitive to ribavirin in cell culture and produced a typical hemorrhagic disease pattern when reinoculated into guinea pigs.

Thus, the efficacy of ribavirin in these models is restricted to protection against the acute, hemorrhagic phase of the infection, and not to the late neurological sequelae. This probably relates to the relative inefficiency with which ribavirin crosses the blood/brain barrier (FERRARA et al. 1981). Since the pathogenesis of the neurological disease observed in humans may differ from that in experimental models (Sect. 4), this may not be relevant to therapy of human AHF. In any case, these studies support cautious application of ribavirin to prophylaxis and therapy of South American HF, with the understanding that the drug should not be expected to be highly effective against CNS virus replication.

5.2 Immunoglobulin

Convalescent plasma has been administered to patients with arenaviral HF for years. MAIZTEGUI et al. (1979) first showed conclusively that such passive immunoglobulin (Ig) therapy was successful in the treatment of AHF, provided that it was given within the first 8 days of illness. The decline in mortality from 10%–30% in untreated cases to less than 1% was accompanied by the appearance of a late neurological syndrome (LNS) in about 10% of immune plasma recipients. In these patients transient febrile CNS disease of low mortality occurred 4–6 weeks after the acute phase of AHF. The pathogenesis is unclear, but does not seem to be related to donor, severity of disease, or several other variables examined. Increasing neutralizing antibody titer of infused plasma enhances survival from the acute disease, but neither increases (as one might predict from the Machupo monkey model (Sect. 4.4.4) nor reduces the incidence of LNS (MAIZTEGUI et al. 1979; ENRIA et al. 1984, 1986a, b). We have modeled antibody therapy of AHF in the guinea pig, and results in the acute disease are quantitatively similar to those found in humans (KENYON et al. 1986b). In addition, associated with viral replication in brain, some treated animals develop delayed-onset CNS disease that may be an analogue of the human LNS. The guinea pig model has been used to quantitate the efficacy of human IgG preparations and demonstrate the utility of murine monoclonal antibodies in therapy; it may also allow us to ascertain the determinants of the unique LNS associated with Ig therapy.

In contrast to the AHF situation, convalescent plasma has not been shown to be beneficial in Lassa fever, even when evaluated systematically (MCCORMICK et al. 1986). Studies of neutralization of Lassa virus in cell culture and therapy of realistic animal models have predicted that this approach would fail and have provided criteria to select a reasoned approach to Ig treatment of human disease.

Humans and other animals surviving Lassa fever develop neutralizing antibodies, but these differ greatly from the ones following Junin or most other

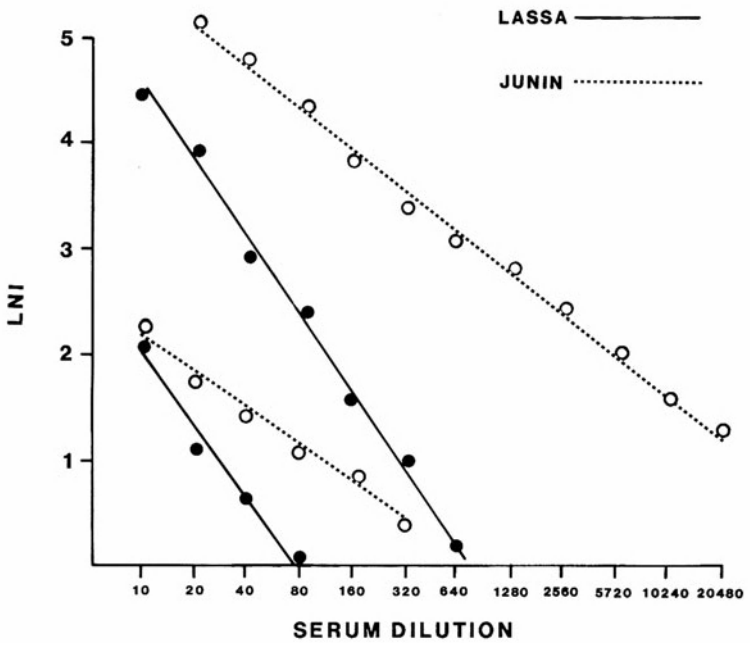

Fig. 7. Neutralization of Lassa (●) and Junin (○) viruses as a function of serum dilution. Twofold dilutions of human convalescent plasma (initial dilution of 1:10) were prepared in diluent containing 10% guinea pig serum and incubated with equal volumes of homologous virus (i.e., Lassa strain Josiah or Junin XJ clon 3) for 1 h at 37°C. LNI were calculated as detailed in Fig. 1 legend. One high-titered and one moderate-titered serum were tested for each virus

viral infections. First of all, significant neutralization appears only after weeks of convalescence (Fig. 4). Secondly, activity is disproportionately reduced by dilution (Fig. 7). Each twofold ($0.3 \log_{10}$) dilution resulted in a fivefold ($0.7 \log_{10}$) decrease in titer. For comparison, the neutralizing capacity of Junin-immune plasma fell in proportion to its dilution factor. This has led to the routine use of a modified test in which reduction in virus titer is the criterion of activity (LNI) rather than the usual serum dilution neutralization test. Thirdly, neutralizing capacity of Lassa convalescent antibodies is highly C dependent (PETERS 1984). Addition of fresh normal serum as a C source restores some, but not all, of the neutralizing titer. As expected, Fab'_2 fragments have no in vitro neutralizing capacity.

The biological significance of these results is exemplified in Table 14, where guinea pig plasma obtained at different times after infection with Lassa virus is compared for its protective efficacy. Within 32–45 days of infection, the animals recovered and had high IFA titers, but neutralizing antibodies were not detected, and plasma did not protect in passive transfer experiments. By 90–180 days plasma from these animals neutralized virus in cell culture (LNI 3.1–3.8) and also prevented death when infused into infected recipients. The importance of LNI is further emphasized by experiments utilizing Lassa strains from geographically different areas, which differ in their neutralization char-

Table 14. Protective efficacy of convalescent guinea pig plasma for Lassa virus-infected strain 13 guinea pigs ($n=10$/group)[a]

Pool	Immune plasma pool infused[b]				Percentage survival	MTD[c] (range)
	Days convalescence	Dilution	IFA	LNI		
1	32	0	2560	0.3	0	18(15–21)
		1:3	640	0.1	0	17(14–19)
		1:9	160	0.1	0	17(14–21)
2	45	0	2560	0.9	0	21(19–24)
		1:3	320	0.6	0	24(16–22)
		1:9	160	0.3	0	21(19–22)
4	90	0	2560	3.4	100	
		1:3	640	3.1	100	
		1:9	160	2.0	90	19
		1:27	40	1.4	50	17(16–17)
5	180	0	2560	3.8	100	
		1:3	640	2.8	100	
		1:9	320	2.2	80	25(24–26)
		1:27	40	1.5	30	21(12–32)
		1:81	10	0.8	0	23(19–27)
Normal plasma					0	17(15–19)

[a] Groups of 10 animals infected s.c. with 3.4 $\log_{10}$ PFU Lassa virus, strain Josiah
[b] 6 ml/kg of convalescent guinea pig plasma diluted in whole, fresh guinea pig plasma, inoculated i.p. into recipient guinea pigs on days 0, 3 and 6 after virus inoculation
[c] Mean time to death in days (JAHRLING 1983)

acteristics. Homologous plasma, with its higher LNI, is more efficacious in guinea pigs (Table 15) or monkeys (JAHRLING and PETERS 1984). Numerous experiments using homologous monkey or guinea pig systems or infusing human antibody into monkeys or guinea pigs suggest that protection can be achieved with doses on the order of 10 ml/kg, provided the LNI exceeds 2.0. In every case, successful treatment of experimental Lassa fever was associated with a suppression of viremia to values at least 100-fold lower than those of controls and often to undetectable levels. No evidence of enhancement of disease was identified, as judged by viremia after plasma injection, survival time, organ viral titers, or histopathology.

If these considerations are extrapolated to human therapy, the neutralizing antibody content of convalescent plasma becomes a limiting consideration. When six Liberian patients were bled sequentially, only two developed a LNI greater than 2.0, and they required more than 6 months to achieve this level. Of 26 donors tested 8 months or longer after illness, only 4 had titers between 2.0 and 3.0 and an additional 4 had titers greater than 3.0 (JAHRLING et al. 1985b). Collection of convalescent plasma in Sierra Leone yielded only 20% of 170 units with an LNI of 2.0 or greater. By using previous titers as one criterion for donor selection, an active plasmapheresis program in the endemic region of Liberia has increased this to 54% of 279 units with titers of at least

Table 15. Protection of strain 13 guinea pigs by convalescent human plasma[a] (from JAHRLING and PETERS 1984)

Lassa viral[b] strain (origin)	Human plasma LNI[c]	ml/kg[d]	% Survival ($n=10$)	MTD (range)
Josiah (Sierra Leone)	1.6	3	0	17.4(15-20)
		6	50	26.0(22-30)
		12	100	–
		None	0	18 (15-20)
Z-132 (Liberia)	2.8	3	80	24 (22-26)
		6	100	–
		12	100	–
		None	0	17.2(15-19)

[a] Human plasma (JM) obtained from a convalescent patient infected with Lassa virus in Liberia

[b] 4.0 $\log_{10}$ PFU Lassa (strain Josiah) or 4.3 $\log_{10}$ PFU Lassa (strain Z-132) inoculated s.c.

[c] LNI vs challenge Lassa viral strain

[d] ml/kg of undiluted plasma inoculated day 0, and repeated days 3 and 6

2.0, but only 15% have a titer in excess of 3.0. Thus, if the quantities of Ig established for effective therapy in experimental models are valid for humans, simple use of unselected convalescent plasma cannot be effective. Indeed, in Sierra Leone, two units of plasma selected on the basis of IFA titers had no appreciable benefit alone or in combination with ribavirin (MCCORMICK et al. 1986).

For this reason we have examined several methods for fractionation and concentration of IgG from human plasma. As Lassa virus neutralizing activity in vitro is C dependent and apparently sensitive to heat denaturation, and because of the requirements for large amounts of IgG for in vivo protection, we were particularly interested in column chromatography techniques producing high yields of native Ig (CONDIE 1979a). In particular, SiO_2 (Aerosil) treatment followed by QAE ion exchange chromatography has been used successfully to prepare intravenous IgG for safe therapy of life-threatening human cytomegalovirus infections (CONDIE et al. 1979b). We have repeatedly fractionated ineffective or marginally effective plasma by either column chromatography or Cohn-Oncley techniques to produce IgG that is protective in monkeys and guinea pigs (CONDIE et al., unpublished observations). In a typical experiment (Table 16) pooled human convalescent plasma with a LNI of 1.6 failed to protect nonhuman primates from Lassa fever. The higher dose (18 ml/kg) suppressed viremia transiently, prolonged the mean time to death, but resulted in only one survivor of four monkeys treated. In contrast, monkeys treated with IgG purified and concentrated from the same plasma pool all survived and their viremias were totally suppressed. These monkeys did eventually seroconvert, however, indicating that they sustained totally inapparent infections. IgG prepared by several methods gave similar activities in neutralization tests and in protection, except for Fab'_2, which was not biologically active, although its antigen-binding capability in IFA was retained.

Table 16. Protection of cynomolgus monkeys against virus[a] by human immune plasma or plasma concentrates

Treatment	LNI	Dose (ml/kg)[b]	Dead/ total	Day of death	Viremia $\log_{10}$ PFU/ml	
					Day 7	Day 14
Plasma[c]	1.6	6	3/4	21, 24, 29	<0.7	4.6
		3	2/2	16, 23	2.3	4.7
QAE[d]	3.0	6	0/3	–	<0.7	<0.7
CM[e]	3.5	6	0/2	–	<0.7	<0.7
None	–	–	4/4	11, 13, 13, 15	3.5	4.0

[a] 4.1 $\log_{10}$ PFU of Liberian strain Z-158 given s.c. This virus has a cumulated mortality of 12/14 untreated cynomolgus monkeys

[b] Days 0, 3, 6, inoculated i.v.

[c] Plasma was collected from Liberian donors, pooled, and aliquots were fractionated by the method indicated (R. CONDIE, pers. comm.). The original plasma pool contained 12.6 mg/ml IgG

[d] Plasma was treated with calcium chloride and Aerosil, then dialyzed to pH 6.6, concentrated, and chromatographed over a QAE A-50 column at pH 6.6. Final IgG concentration was 25.6 mg/ml

[e] Plasma was treated with calcium chloride, then dialyzed to pH 5.0, concentrated, and chromatographed on carboxymethylcellulose (CM)-Sepharose at pH 5.0. Final IgG concentration was 25.8 mg/ml

Thus, IgG therapy of Lassa fever appears to be feasible in principle, although acquisition and processing immune plasma for general use in endemic regions of Africa are impractical. The testing of intravenous IgG preparations in the field is still worthwhile, since ribavirin therapy is not life-saving in some Lassa patients and since the information will facilitate the rational design of therapeutic regimens utilizing protective monoclonal antibodies as well as vaccine development.

5.3 Future Approaches

Existing therapy of arenaviral HF is effective, and it should be improved in the near future (PETERS and SHELKOV 1986). Use of the neutralizing antibody test as the critical measurement to predict therapeutic efficacy in the contrasting Lassa and Junin situations has led to a proper perspective on the use of convalescent plasma in the treatment of humans. It is not, however, entirely clear whether these glycoprotein reactive antibodies function through actual virus neutralization or whether programming infected cells for lysis by other effector mechanisms may be an auxiliary or even dominant action. Furthermore, there may be other protective epitopes not yet identified. These problems will become more acute as highly specific monoclonal antibodies are developed as standardized treatment modes, to avoid problems of supply and of blood-borne disease; hopefully these monoclonal antibodies will provide a mechanism to answer the same questions they raise.

Ribavirin is a highly effective antiviral drug, but it has definite limitations in the therapy of CNS disease in several animal systems and when applied

late in the course of human illness. Numerous congeners have been tested without improving on the original drug, but it may still be possible to bind ribavirin to other compounds to facilitate entry into the CNS. Broadening antiviral drug screening targets to include arenaviruses (e.g., Pichinde with only P2 containment requirements) has already resulted in the identification of compounds with promise in cell culture and possibly experimental animals (HUGGINS et al. 1984).

Combination virus-specific therapy also clearly has a place. In the nonhuman primate model for Lassa fever, successful treatment can be achieved with ribavirin and antibody after it is no longer possible with either alone. This is clearly the next avenue to pursue in reducing morbidity and mortality.

Other approaches should rely on avenues that are not virus-specific, but rather attempt to combat specific physiological derangements induced by the virus the (Sect. 3). Application of concepts evolving from the study of "shock lung", myocardial depression seen in other shocklike states, endothelial and platelet physiology, and participation of several specific soluble mediators are all pertinent. Pharmacological therapy may be improved through these avenues, and an excellent candidate may be desmopressin (SALZMANN et al. 1986; KOBRINSKY et al. 1984). This compound improves vascular integrity through its action on the von Willebrand factor and is effective in states where this is not the specific defect.

6 Vaccines

6.1 Lassa

The major health impact of Lassa fever in West Africa poses an urgent requirement for vaccine development. While it was recognized at the outset that the immunobiology of Lassa fever in humans and experimental animals was only partially understood, inactivated Lassa viral concentrates and naturally attenuated arenaviruses (e.g., Mopeia and Mobala) were reasonable starting points for initiating vaccine development. Neither approach can be considered successful. In our laboratory we prepared Lassa viral concentrates and inactivated them by several different preocedures (Table 17). Although all of these "vaccines" elicited antibodies measured by IFAT, none induced neutralizing antibody, and none protected guinea pigs against lethal challenge. Viremias in challenged animals were not significantly delayed or reduced in magnitude (data not shown), and mean times to death were not significantly delayed. Other investigators employing a gamma-irradiated preparation in conjunction with pertussis as adjuvant were able to prime monkeys to respond to all three Lassa viral structural proteins, NP, GP1, and GP2, but also failed to obtained protection (MCCORMICK, pers. comm).

Since high titers of infectious virus were present before virus inactivation, and since three different inactivation procedures were used, it seems unlikely that the direct approaches to killed viral vaccines which have succeeded with other agents will be applicable to Lassa fever.

Table 17. Protective efficacy of inactivated Lassa viral vaccines[a] in strain 13 guinea pigs

Method of inactivation	Number of injections[b]	Adjuvant	Results of immunization and challenge[c]			
			IFA	LNI	Dead/ total	MTD (days)
0.05% formaldehyde	3	CFA	320	<0.3	10/10	18.2
Psoralen and UV	2	CFA	160	<0.3	10/10	16.4
Gamma irradiation	3	none	160	<0.3	10/10	17.5
Gamma irradiation	3	CFA	1280	<0.3	10/10	19.5
No vaccine	–	–	<10	<0.3	10/10	15.3

[a] Vero cell culture supernatant concentrates. Preinactivation titers: 8.7–9.4 $\log_{10}$ PFU/ml
[b] 0.4 ml "vaccine" plus 0.4 ml Freund's complete adjuvant, inoculated s.c. on days 0, 14, and 28
[c] Guinea pigs were challenged with 4.0 $\log_{10}$ PFU Josiah strain Lassa virus s.c. 45–60 days after the third immunization dose

Two Old World arenaviruses, Mopeia and Mobala, are highly attenuated for macaques and guinea pigs and provide solid protection against Lassa challenge (Sects. 2.3 and 4.2). It has been suggested that they are naturally occurring attenuated vaccine candidates, but there are no data on their pathogenicity for humans. Furthermore, Mopeia, the better studied of the two, persists in monkey spleens for long periods after inoculation. Some reports also suggest that Mopeia-infected macaques may have significant histopathological lesions in the liver or kidney and may also develop lesions after Lassa viral challenge (LANGE et al. 1985). Development of a classical live attenuated vaccine from Lassa virus itself poses formidable problems in human testing, particularly in assuring that latent virus infection would not pose an unacceptable risk.

The uncertainties of live arenaviral immunization and the failure of prototype inactivated viral concentrates may ultimately demand that alternative approaches be undertaken. These should allow the flexibility to analyze the critical epitopes and optimum presentation to assure a protective humoral and/or cellular immune response and will permit inoculation of either subgenomic or nucleic acid-free immunogens. These approaches include the development of synthetic peptides, anti-idiotypic antibodies, and vaccinia constructs containing genes for Lassa viral structural proteins.

6.2 Machupo

Initial attempts to develop a live, attenuated Machupo vaccine candidate focused on suckling mouse brain-passaged material. Investigators at the Middle American Research Unit passaged prototype Carvallo virus 80 times in suckling mouse brain (JOHNSON 1975). This material did not produce detectable viremia or clinical signs in marmosets and rhesus monkeys, but resulted in low (1:8) or undetectable neutralizing antibody responses. Two rhesus macaques inoculated with 10^3 PFU of the attenuated virus were protected against a lethal challenge with virulent Machupo (EDDY, unpublished observations).

Attenuation of Machupo via cell-culture adaptation proved more difficult (EDDY, unpublished observations). Following isolation of virus from human spleen in certified fetal rhesus lung cells, serial passage in chick embryo cells yielded indications of progressive in vitro adaptation and attenuation for guinea pigs. Continuation through passage 31 demonstrated progressive, though insufficient, attenuation for rhesus monkeys as well. With additional passage, however, reversion to virulence was observed and further studies were abandoned when no improvement was seen after 60 passages.

Alternative efforts to develop an experimental killed Machupo viral vaccine were only marginally more successful (EDDY, unpublished observations). Despite initially encouraging results with BHK-21-derived virus, consistent problems with obtaining adequate yields in diploid cell cultures precluded further studies.

6.3 Junin

Soon after the isolation of Junin viruses, formalin-inactivated vaccines were developed (PIROSKY et al. 1959). This early mouse brain immunogen was administered to more than 15000 people between 1959 and 1962, but systematic studies of the results have never been reported (METTLER 1969). The use of formalin- or other inactivated products (BARRERA ORO et al. 1967; PARODI et al. 1965; MARTINEZ-SEGOVIA et al. 1980) was shown to be feasible in laboratory studies, but required multiple inoculations of high-titer starting material and only protected laboratory animals against low challenge doses of Junin virus. Because viral titers achieved in cell strains suitable for vaccine production were low, attempts to produce a classical, inactivated Junin immunogen were abandoned.

Cross-protection against Junin virus by serologically related Tacaribe virus (PARODI and COTO 1964; TAURASO and SHELOKOV 1965; Sect. 4.2) has been the basis for suggesting Tacaribe virus as a potential live heterologous vaccine against AHF (COTO et al. 1967; WEISSENBACHER et al. 1982). Although Tacaribe virus is nonpathogenic for guinea pigs and nonhuman primates and is nonneurovirulent for the marmoset *C. jacchus* (SAMOILOVICH et al. 1984), its potential for human disease is unknown. The only recorded infection of man was accompanied by fever, headache, and moderately severe constitutional symptoms (BISHOP and CASALS, pers. comm.). As there is a lack of information on the consequences of human infection with Tacaribe virus, this interesting approach still has not gained wide acceptance.

The first live-attenuated Junin vaccine (XJ clone 3) was developed from a plaque-cloned mouse passage of prototype XJ Junin virus. The candidate was attenuated for guinea pigs and therefore was tested in at risk laboratory workers (GUERRERO et al. 1969). Doses ranging from 3.7–5.0 $\log_{10}$ $SMICLD_{50}$ provoked mild clinical reactions followed by rapid recovery (RUGIERO et al. 1969) and the development of neutralizing antibodies (WEISSENBACHER et al. 1969). Eventually 636 volunteers received this vaccine. About one-fourth of the recipients had no clinical or laboratory abnormalities, while another one-fourth had low-grade fever often accompanied by myalgia, leukopenia, and thrombocytopenia (RUGGIERO et al. 1974). Follow-up studies 7–9 years later revealed no long-term

clinical side effects; 90% of those vaccinated still had Junin viral neutralizing antibodies (RUGGIERO et al. 1981).

Administration of XJ clone 3 to humans was halted because of its incomplete passage history, its prior passage through heteroploid cells, and its method of preparation (suckling mouse brain). More recent investigations of the residual pathogenicity of XJ clone 3 and other attenuated passages of XJ (BOXACA et al. 1982; LAGUENS et al. 1983b; MALUMBRES et al. 1984; GUERRERO et al. 1985) indicate they would not pass some of the animal safety tests required by the Food and Drug Administration for human vaccines (Code of Federal Regulations 1985).

Candid −1, another attenuated XJ derivative, was developed to circumvent the problems of previous vaccine candidates (BARRERA ORO and EDDY 1982). The already attenuated 44th suckling mouse brain passage of XJ was passaged into vaccine-certified FRhL−1 cells and shown to be free of adventitious agents. It was cloned by oligoburst (WALEN 1963) and limiting dilution passages. Advantage was taken of mouse, guinea pig, and rhesus models of virulent AHF to demonstrate extensive attenuation and loss of neurovirulence (CONTIGIANI and SABATTINI 1977; Sect. 2.6). Candid −1 was stably attenuated on in vitro and in vivo passage (indeed, significantly more attenuated than XJ clone 3) and passed all required safety tests, including i.c. inoculation of rhesus monkeys. The protective $dose_{50}$ for either guinea pigs or rhesus monkeys challenged s.c. with virulent Junin strains was less than 30 PFU, and guinea pigs were protected from aerosolized virus.

Candid −1 was assayed in four human volunteers (2.8×10^4 PFU s.c.) and produced no significant clinical or hematological alterations. However, the lack of reactogenicity was accompanied by a low humoral immune response; only three of the recipients developed low-level neutralizing antibodies. To date 17 human volunteers have received Candid −1 without significant clinical or laboratory reactogenicity. Neutralizing antibodies developed in 85%. Most recipients have antigen-specific lymphocyte proliferation, even in the absence of detectable antibody (KENYON, unpublished observations). Studies in additional volunteers and assays of cellular immunity are underway to extend these observations.

7 Conclusions

The models presented here were chosen to illuminate the problem of naturally acquired arenaviral HF, and many of the conclusions contrast with those drawn from the study of mice infected with laboratory strains of LCMV. Perhaps the most striking difference is the lack of any reliable evidence for immunopathology mediated by either T-lymphocytes or circulating immune complexes. Multiple immunosuppression experiments have failed to spare animals from fatal arenavirus HF. Although the adequacy of immunosuppression can always be argued, several measures of the immune response were rendered undetectable, and the same regimens converted asymptomatic, immunizing infections with attenuated viral strains into lethal disease. Similarly, the primacy of T cells (JOHNSON et al. 1978) and perhaps even more specifically cytotoxic T cells

(BYRNE and OLDSTONE 1984; BAENZIGER et al. 1986) in viral eradication in the LCMV-infected mouse is not as clear-cut in VHF models. This may be an erroneous conclusion because of the necessarily less sophisticated immunological analysis possible in guinea pigs and primates, or it may reflect virus-host differences.

The common denominator for recovery in the models of viral HF appears to be an effective immune response to infected cells rather than activation of a particular arm of the immune response. Available evidence in the Lassa-LCM systems suggests that cytotoxic or delayed hypersensitivity T cells are responsible, while in the Junin-infected guinea pig, antibodies to viral glycoprotein, in concert with complement or activated Fc receptor-bearing cells, seem to dominate. Protection against infection with homologous or heterologous viruses can be mediated either by cellular immune mechanisms or by circulating B-cell products. Prior infection with an Old World arenavirus or the New World Tacaribe virus protects against virulent Lassa or Junin challenge, respectively. Cell-mediated effector mechanisms are implicated in the case of Lassa virus by the ineffectual neutralizing antibody response, by classical cell transfer studies in inbred guinea pigs, and by the correspondence of patterns of protection and antigenic specificity of in vitro cytotoxicity tests. The same mechanism probably holds true for Junin, although a high-titered anamnestic neutralizing antibody response somewhat clouds the issue.

The late onset of the immune response to arenaviruses, particularly the unusual properties of the neutralizing antibodies to Lassa and LCM viruses, suggests that even clinically mild arenavirus infections may have important effects on the immune system. Global immunosuppression seems to be a feature of clinically severe disease, and failure to mount an effective virus-specific immune response is characteristic of fatal infections. It may be mediated by interferon, macrophage infection, or other mechanisms.

The outcome of infection in these arenaviral HF models depends on virus strain, host genotype, and the status of the host. It has become clear that there is considerable variation in the pathogenicity of different isolates of the "same" virus. In the case of Lassa and Junin viruses, there has been a correlation with the human disease syndrome from which the isolate was made. In experimental animals less virulent viruses usually replicate to lower titer in organs and cause lower viremias than their more virulent counterparts. Immunosuppression does not change the initial pattern of replication, but may allow them to eventually achieve higher titers and become lethal for the host. In other situations virus replicates at a high level in the host from the beginning, but about 7–10 days after inoculation, viremia begins to decrease in those destined to survive; this mechanism is also sensitive to immunosuppression, and in our experience is particularly common in situations where host genotype determines resistance to viral infection.

The lack of sufficient histological evidence to explain the lethal outcome of arenaviral HF led us to analyze the Pichinde-infected guinea pig from the physiological perspective (LIU et al. 1982, 1983). Marked weight loss, cardiac dysfunction, increased vascular permeability (particularly in the lung), and renal electrolyte abnormalities resemble those seen in human disease and should form

a basis for improving clinical management of specific defects. Soluble mediators have been identified as important in the disease process: α-interferon in many of the symptoms of AHF, leukotrienes in the cardiac and vascular abnormalities of Pichinde-infected guinea pigs, and platelet activation with prostacyclin-TxA2 abnormalities in the increased vascular permeability of primate Lassa fever. Understanding the control and interrelations of these and other mediators in the pathophysiology of HF is one of the major avenues to improving therapy.

Although hemorrhage is a dramatic and characteristic accompaniment of the HF syndrome, abnormalities of vascular regulation and permeability are more fundamental. A decrease in platelet number or function is probably a major determinant of frank bleeding from a damaged vascular bed. Clotting abnormalities probably result from multiple causes, with disseminated intravascular coagulation occurring only in a minority of cases and not being important in the pathogenesis of the basic disease.

One of the most characteristic differences between the South American Hfs and Lassa fever is the almost universal occurrence of neurological signs during the course of the former and the less frequent CNS involvement in the latter. The models discussed here uniformly demonstrated the prominent neuroinvasiveness and neurovirulence of Junin and Machupo viruses. This contrasts with the lack of direct virological evidence for viral infection of the human CNS in South American HF. The resolution of the pathogenesis of the CNS involvement is important for vaccine development, antiviral drug applications, and understanding of the late neurological syndrome observed after plasma treatment of AHF.

Specific therapy of arenaviral HF is surprisingly advanced compared to many better-understood viral diseases. Convalescent plasma is known to be highly effective in AHF, as is the antiviral drug ribavirin in Lassa fever. Realistic models of HF were essential to the development of ribavirin therapy and will continue to contribute to assessment and discovery of newer modalities. For example, combined ribavirin and IgG treatment of Lassa fever has been shown to be superior to either alone in primate models of Lassa fever, monoclonal antibodies to Junin virus prevent AHF in the guinea pig, and newer antivirals are in preclinical testing. The marriage of in vitro neutralizing antibody tests and in vivo observations has been critical in understanding the basis for antibody therapy of these diseases. The amount of antibody needed for successful treatment of AHF can now be quantified; similar considerations in animal systems predicted the failure of human convalescent plasma therapy and provided a rational approach to treatment with larger quantities of IgG.

Vaccination against Lassa fever and AHF is a desirable goal, since intervention in human life styles and ecological conditions are unlikely to prevent natural infections. A live attenuated vaccine for AHF has been developed and is under human testing. It should have utility in the relatively small, prosperous region of Argentina where the population at highest risk resides. Classical vaccine technology has failed to develop other successful live or inactivated candidate immunogens, although alternate approaches are actively under development. Progress to date has leaned heavily on the use of guinea pig and primate models, and future studies will be equally dependent.

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Epidemiology and Control of Lassa Fever

J.B. McCormick

1 Introduction

The history of recognized human arenavirus infection in Africa began in 1969 with the mysterious death of two medical missionaries and the near-fatal illness of a third (Frame et al. 1970). An arenavirus was isolated from two of these patients and given the name of Lassa virus after the town of Lassa, Nigeria, where the disease, known as Lassa fever, occurred (Buckley et al. 1970). An earlier description of a febrile syndrome in Sierra Leone was, in retrospect, very likely to have been the first account of Lassa fever; however, no etiologic agent was sought (Rose 1956, 1957).

Subsequently, the disease has been described in several different West African countries (Monath et al. 1973, 1974a; Carey et al. 1972; Fraser et al. 1974).

Much of the initial epidemiologic information about Lassa fever was from investigations of hospitalized patients, many of whom had infection of nosocomial origin. Most instances were attributed to person-to-person transmission of the virus (Frame et al. 1970; Carey et al. 1972; Monath et al. 1973; Fraser et al. 1974; Bowen et al. 1975; Frame 1975) which is not a feature of the other known human arenavirus infections. Indeed, it was transmission to hospital staff that led to the discovery of Lassa fever.

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The fact that Lassa virus was an arenavirus suggested the existence of a rodent host, which was identified when Lassa virus was isolated from *Mastomys natalensis* in 1972 (MONATH et al. 1974b). Our knowledge of African arenavirus epidemiology has now been expanded by the isolation of viruses related to Lassa virus from a number of rodent species from other parts of Africa (Central African Republic, Zimbabwe, Mozambique, and South Africa), although their roles in human disease remain undefined (WULFF et al. 1977; JOHNSON et al. 1981; GONZALEZ et al. 1983; KEENLEYSIDE et al. 1983).

The relative importance of person-to-person and rodent-to-person transmission in the natural history of Lassa fever in rural West Africa has not been satisfactorily assessed. Epidemiologic studies that would allow independent identification of either mode are difficult to design. The single study thus far attempted indicated that person-to-person spread occurred in villages, but its relative importance could not be accurately determined (KEENLEYSIDE et al. 1983).

Thus, the epidemiology of Lassa fever has two principal themes: rodent-to-human spread, which appears to predominate, and person-to-person spread, which is also of importance. Intuitively, the spread to humans from a chronically infected rodent host would appear to be the fundamental mode of transmission, and certainly the main reservoir is the rodent. It is thus natural to begin a description of the epidemiology of Lassa fever with a discussion of the ecology of its rodent host.

2 Rodent Ecology

The only known reservoir of Lassa virus in West Africa is *Mastomys natalensis,* one of the rodents found most commonly throughout the entire African continent. Although many other species of West African rodents have been studied, none have been found to harbor the virus. There exist at least three different species of *Mastomys,* with different diploid types; 32, 36, and 38 chromosomes (MATTHEY 1958; HALLETT 1979; ROBBINS et al. 1983). The 32 and 38 types occur in West Africa, whereas the 32 and 36 are found in southern Africa. All three types are known to be reservoirs of Lassa virus (32 and 38) or related viruses (e.g. Mopeia virus) (36).

The two species of *Mastomys* in Sierra Leone occupy somewhat different ecologic niches. In the eastern province, mostly an area of secondary forest with very high rainfall, the 32-chromosome species predominates and is found primarily in houses, not in the surrounding bush or agricultural areas (Table 1). Both 32- and 38-chromosome species occur in the northern savannah areas, but here the 38-chromosome species predominates; it is found more often in the bush and agricultural areas, in contrast to the 32-chromosome species located in houses, as in the eastern province. Both species in the wild are more or less equally infected by Lassa virus (Table 2) (MCCORMICK et al. 1985). In the laboratory in utero infection and horizontal infection postpartum can be demonstrated in both species, resulting in lifelong excretions of large quantities of Lassa virus (J.B. MCCORMICK, S.B. BAUER, unpublished observation). Studies of *Mastomys* in the villages of Sierra Leone have shown that there is little

Table 1. Percentage of *Mastomys natalensis* in houses and surrounding bush of two villages in eastern province, Sierra Leone, compared to a total of all rodents

House	Bush
63.4 (946/1491)	14.3 (52/367)

$P < 10^{-6}$; df, 1

Table 2. Lassa virus and antibody from *Mastomys natalensis by region of Sierra Leone*

	Karyotypes: 2n = 32		Karyotypes 2n = 38	
	North (%)	East (%)	North (%)	East (%)
Virus	3/40 (7.5)	68/699 (9.7)	24/232 (10.3)	1/15 (6.7)
Antibody	4/40 (10.0)	176/771 (22.8)	49/234 (20.9)	0/15 (0)

North, savannah; *east,* secondary forest; indirect fluorescent antibody (IFA) $\geq 1:8$ were considered positive in rodents

movement from house to house, and that their average lifespan is about 6 months (J.W. KREBS, J.B. MCCORMICK, unpublished observation). However, most studies of *Mastomys* have been conducted over a relatively short (1- to 2-year) period, and perhaps a longer investigation may reveal wider fluctuations in domestic *Mastomys* movement resulting in their appearance in or disappearance from a given house. The breeding patterns of *Mastomys* do not show significant seasonal fluctuation, thus, the population appears stable on the average in a given village over at least a 1- or 2-year period. The conclusion from these observations is that the primary place of contact between humans and *Mastomys* must be in and around village houses.

Indeed, one can paint an appealingly complete picture for the rodent-human contact. The houses are often closed up during the day, creating almost continuous darkness for these (essentially nocturnal) rodents. Also the human residents are usually out of their houses in the daytime so that rodents may deposit urine extensively, day or night, almost anywhere including floors, beds, and food areas. The urine may contain 10^3–10^5 tissue culture infective dose $(TCID)_{50}$/ml of Lassa virus, and the relative stability of Lassa virus in the environment allows frequent human contamination.

3 Human Epidemiology in West Africa

3.1 Seasonality and Severity

Following its discovery in Nigeria, Lassa fever was quickly recognized and identified in other parts of West Africa. Recent studies in Sierra Leone have

suggested that it is a very common cause of hospitalization accounting for 10% to 15% of adult medical admissions and 30% of related adult deaths in two hospitals surveyed (McCormick et al. 1987). The fatality rate of these hospitalized patients was 16%, with no age or sex differences. Of 245 febrile children from whom adequate specimens were obtained, 51 (21%) had Lassa fever (Webb et al. 1986). Virus was isolated from 23 of these children. In this group, significantly higher percentage of febrile female children had Lassa fever than male children. A similar observation was made from serologic studies described later in this chapter.

Pregnant women appear to be particularly at risk of severe infection. Studies have shown that Lassa fever is the most common cause of septic abortion in the study hospitals, although the overall mortality of the mothers is not significantly higher than for other patients hospitalized with Lassa fever (Price et al. 1986). However, infection in the third trimester of pregnancy is associated with a higher mortality compared with infection earlier in pregnancy.

Long-term hospital-based studies have shown that Lassa fever infections occur throughout the year in secondary forest areas where the highly commensal 32-chromosome *Mastomys* predominates. There is a relative increase in the number of Lassa fever cases during the dry season months of January–May (Fig. 1). This may be related to the longer survival of the virus at lower humidity (Stephenson et al. 1984), since there is no evidence for fluctuations in the *Mastomys* population or major changes in human behavior during the dry season.

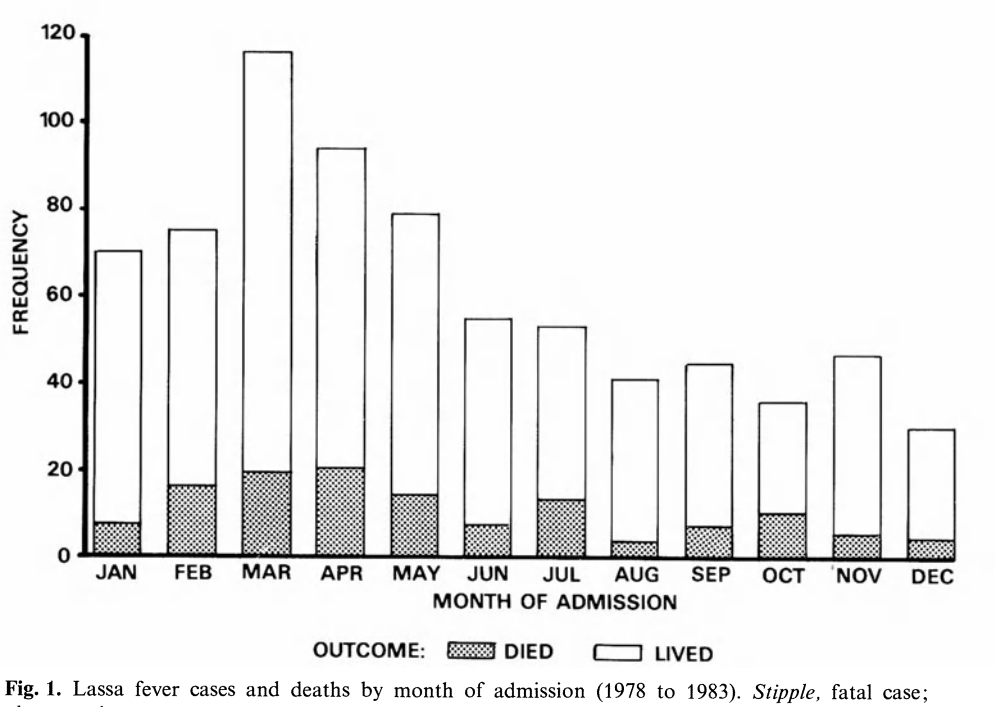

Fig. 1. Lassa fever cases and deaths by month of admission (1978 to 1983). *Stipple*, fatal case; *clear*, survivor

Indeed, the people spend more time inside houses during the rainy season than during the dry season. Seasonal data are not available from hospitals in the drier savannah areas. The conclusion from these studies is that Lassa fever occurs as an endemic disease and accounts for a high proportion of febrile illnesses in endemic areas, where the incidence increases in the dry season.

3.2 Nosocomial Transmission

The principal published data on human-to-human transmission are from early investigations of hospital outbreaks (FRAME et al. 1970; MONATH et al. 1974a; CAREY et al. 1972; FRASER et al. 1974). These reports suggest that the most important means of nosocomial transmission is close contact with patients' blood and secretions, via minor cuts or abrasions in the skin, needle sticks, or trauma from other medical instruments. Only one instance of suspected aerosol transmission has been reported (CAREY et al. 1972), and this conclusion stemmed primarily from lack of evidence that several patients had direct contact with the index case. It is difficult to document patient contact in African hospitals where the movement of patients and visitors is uncontrolled. Such conclusions in these circumstances could be misleading. Patterns of disease in village settings do not suggest aerosol transmission; similarly, spread by insects is unlikely on epidemiological grounds. Recent prospective studies of seroconversion rates to Lassa virus-positive among hospital workers in an endemic area of Sierra Leone showed no significantly higher levels than in the populations of adjacent villages. No specific risk group was identified within the hospital personnel, many of whom lived in rat-infested areas. It was clear, however, that seroconversion rates were significantly higher in hospitals that did not use basic barrier nursing techniques (gowns, gloves, and masks) for the care of Lassa fever patients compared with hospitals that did (HELMICK et al. 1986).

3.3 Prevalence of Antibody to Lassa Virus

Serologic surveys in Sierra Leone have shown that Lassa fever is a local disease with considerable variability in antibody prevalence from village to village. There is also a geographic difference, with coastal areas having lower prevalence than secondary forest and savannah areas (MCCORMICK et al. 1987b). Recorded ranges are from 8% to 50% in villages in different areas of Sierra Leone. More extensive studies of Lassa fever were performed in many villages in a highly endemic area of Sierra Leone's eastern province (Table 3) (MCCORMICK et al. 1987b). The distribution was wide (10%–50%), with no specific factors identified that were associated with high or low antibody prevalence in the population. Age- and sex-specific analysis (Table 4) showed no detectable antibody in children under the age of 1 year; females under the age of 15 had a higher prevalence of antibody than did males of the same age group, corresponding to the higher frequency of cases in female children already described. No differences were seen between the sexes above the age of 15 years. Although

Table 3. Prevalence of antibody[a] to Lassa virus in Eastern Sierra Leone

Village	Sample	Human antibody prevalence (%)
Palima	217	52
Semewabu	372	38
Konia	733	37
Tongola	402	31
Niahun	1075	19
Kpandebu	578	15
Lowoma	285	13
Bomie	617	10

[a] IFA titers $\geq$1:16 were considered positive in humans

Table 4. Antibody to Lassa virus by age group in populations of four villages (Sierra Leone)

Age (years)	Positive[a]/tested (%)
>4	25/227 (11)
5–9	92/527 (17)
10–14	69/316 (22
15–19	56/256 (22)
20–29	192/565 (34)
30–39	121/404 (30)
40–49	84/267 (32)
50+	26/157 (17)
Total	684/2810 (24)

[a] *Positive,* IFA titers $\geq$1:16; males, 21% with antibody; females, 28% with antibody

these data might suggest a higher exposure to virus of females, who spend more time around or in the house, such a hypothesis is extremely difficult to examine because efforts at accurate quantitation of time spent in the house have not been successful. There is generally an increase in antibody prevalence with age, suggesting that increased time of exposure in the villages is associated with a higher probability of infection. This contrasts with the age and sex distribution of Junin virus infection in Argentina, where the vast proportion of infected persons are adult male agricultural workers. Several studies of antibody prevalence to Lassa virus including occupation, education, and socioeconomic factors have failed to demonstrate any specific association or risk groups. This is probably because many of the basic activities of the population are the same, regardless of their occupation. For example, tailors, store clerks, school teachers, are all also involved in agriculture, and their lifestyle and type of housing are the same.

3.4 Incidence and Disease to Infection Ratios

Prospective studies of seroconversion rates with respect to Lassa fever virus were conducted in several villages in Sierra Leone (McCormick et al. 1987b). These studies showed seroconversion rates ranging from 5% to 20% of the susceptible population per year. The highest rates were seen in diamond mining areas with large, crowded, migrant populations, extremely rapid turnover, and excess food supplies. The lower rates occurred in agricultural villages with more stable populations.

The same prospective studies have also shown disease to infection ratios of from 10% to 25%, and a proportion of all febrile illness due to Lassa fever in the study villages ranging from 5% to 14% (McCormick et al. 1987b).

Estimates of hospitalization to infection ratios are about 5% to 8%, with a fatality to infection ratio of 1% to 2%. Although these data show that Lassa fever is less likely to be fatal than originally estimated (McCormick et al. 1987b), they also point out that the disease is much more common than previously believed; 200000–300000 infections per year with 3000–5000 deaths may be a conservative estimate of the toll Lassa fever takes in all of West Africa.

The prevalence of antibody and the seroconversion rates in various village populations are not easy to reconcile, since seroconversion rates of 20% per year should lead to total population immunity. However, we also observed a high rate of antibody loss in these populations (McCormick et al. 1987b) and evidence of reinfection, as demonstrated by eightfold increases in antibody titers to Lassa virus. These rises in antibody do not appear to be associated with febrile illness, although the number of observations is not large enough to detect rates of illness below 5% (McCormick et al. 1987b). Thus, some of the seroconversions we observed may have been reinfection from individuals who had lost detectable antibody. Consequently, the rate of true primary infection may be much lower. This may also mean that the proportion of the population with true immunity to Lassa fever may be higher than the antibody prevalence suggests.

4 Other Countries in Africa

Human data from four different West African countries, although fragmentary, begin to give a more complete picture of the distribution of Lassa fever than previously available. Although less complete than information for Sierra Leone, data from Guinea and Liberia suggest similar prevalence and geographic distribution. In Guinea antibody levels in the human population are low in the coastal and mountainous areas, but range from 1% to 2% to as high as 25% in the forest and savannah areas (A.H. Demby, J.B. McCormick, unpublished data). Antibody prevalence in Liberia also appears to be widespread in populations living in the forest areas, and reports of the frequency of disease are similar to those in Sierra Leone. Finally, although data from Nigeria are scant, it now seems that the disease is widespread throughout the country, to a much larger degree than previously appreciated. Antibody prevalences of 20%–30% are seen in the populations of the more familiar northern savannah areas, but the southern parts of the country also have a high prevalence (2%–15%), (D. Tomori, A. Fabiye, J.B. McCormick, unpublished data). Scattered reports also exist of human infection by Lassa virus in Burkina Fasso and Mali; however, no systematic studies have been carried out in these countries.

Viruses related antigenically to Lassa virus have been isolated from rodents in the Central African Republic, Zimbabwe, Mozambique, and South Africa (Wulff et al. 1977; Johnson et al. 1981; Gonzalez et al. 1983). Humans with antibodies to these viruses have been reported in the Central African Republic

and Mozambique, but the clinical severity or significance of these infections remains unknown.

Taken together, these observations demonstrate the presence of a complex of antigenically related arenaviruses in Africa associated with several species of rodent hosts, but with disparate potential for human infection and disease, just as has been described for South American arenaviruses. The genetic and antigenic relationships between these viruses are now partially understood. Oligonucleotide mapping has demonstrated 98% base homology between viruses from Nigeria and Sierra Leone, 80% homology between representative viruses from west and central Africa, and 70% homology between representative viruses from central and southern Africa (GONZALEZ et al. 1984). Antigenic relationships allow African viruses to be geographically grouped as west, central, and southern African in origin. Although the classical biological relationship of cross-neutralization has been impossible to demonstrate, due to the inability to neutralize the viruses, earlier studies have demonstrated that Mopeia virus infection may protect monkeys from challenge by Lassa virus (KILEY et al. 1979).

5 Prevention

5.1 Rodent Control

The single most important risk factor so-far identified for acquisition of Lassa virus infection appears to be sustained living in an endemic area. Thus, preventive measures such as rodent control or vaccination would have to be broadly applied in such an area. The general control of an ubiquitous rodent population such as *Mastomys* is not a realistic approach to the control of Lassa virus infection. Studies of the effect of extensive rodent trapping in a village have shown that the level of virus transmission may be reduced by as much as fivefold following removal of $>90\%$ of *Mastomys* (J.B. MCCORMICK, J.W. KREBS, and P.A. WEBB, unpublished data). Identification of a village with high levels of virus transmission warrants serious consideration of a rodent control program in that village. Such a program should focus on the reduction or elimination of rodents in houses, where maximal contact with the animals is likely to occur, rather than in surrounding areas. Outdoor rodent trapping, which is more difficult and much less efficient, is likely to be less effective in reducing virus transmission.

5.2 Vaccine Application

The target population for an eventual vaccine should include hospital workers in endemic areas and the general population in areas with a significant prevalence of antibody to Lassa virus. This would include large populations in West Africa, especially Nigeria, Liberia, Sierra Leone, and Guinea, and possibly popu-

lations of southeast Africa in Mozambique and Zimbabwe, depending on the level of disease associated with human infection in these areas.

6 Summary and Conclusion

Lassa fever is a common disease in West Africa, with an antibody prevalence of 20%–50% in some regions. Transmission occurs primarily from rodent to man, with some person-to-person transmission, but the home appears to be the principal locus of transmission, since *Mastomys* is found primarily in or around human dwellings. Rodent urine appears to be the major source of virus, and the sites of initial infection may be multiple. Aerosol transmission of the virus appears to occur rarely, if at all, and hospital transmission is usually associated with failure to practice barrier nursing techniques. The fatality from all such infections is probably 1%–2%, but the mortality in untreated hospitalized patients is 15%–20%. Control of rodents in houses of highly endemic villages appears to reduce the rate of transmission to humans. Future goals should include education of the population affected regarding rodent control, and the eventual development and application of a vaccine.

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Argentine Hemorrhagic Fever

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1 Introduction

Argentine hemorrhagic fever (AHF), an endemoepidemic disease of viral etiology, is geographically restricted to the central area of the Pampas, in the heart of the richest farmlands of Argentina. Unknown before the 1950s, its salient clinical features are hematologic and neurologic signs, including a febrile syndrome, together with varying degrees of cardiovascular, renal, and digestive alterations, first described in detail in 1955 (ARRIBALZAGA 1955).

Thanks to Parodi's pioneering work (PARODI et al. 1958), closely followed by Pirosky's (PIROSKY et al. 1959), the viral etiology of AHF was firmly established in 1958, when a previously unknown virus was isolated from blood and tissue samples of a patient resident in Junín, a city of Buenos Aires Province.

Although the epidemic zone is small (120000 km^2) relative to Argentina's total area (2800000 km^2), its population density and economic significance, mainly due to agricultural and livestock production, make AHF an important health problem (MAIZTEGUI and SABATTINI 1977).

Up to now, the number of clinical cases reported has ranged from 100 to 4000, totalling 21000 for the 30-year period, with a mean of 360 for the past 5 years. Male rural workers between 20 and 50 years old are particularly affected. Most of them are corn, sorghum, or sunflower harvesters. Incidence peaks coincide with harvest times for these crops between April and July, late fall, and early winter (MAIZTEGUI 1975).

There is also a correlation with local wild rodent density increase, mainly *Calomys* genus (VANELLA 1964), which hardly ever invade urban areas, thus explaining rural AHF prevalence. In nature Junín viurs (JV) is maintained by persistent infection of these animals, which by shedding virus through saliva and urine, achieve horizontal transmission inter se (SABATTINI et al. 1977)

C. laucha and *C. musculinus* are the main *Calomys* genus species acting as reservoirs. Their geographic distribution has not yet been strictly delineated, but they are known to spread over at least two-thirds of the Argentine territory, much greater than the endemic zone. On occasion, JV and /or anti-JV antibodies have been detected in rodent species such as *Mus musculus*, *Akodon azarae*, and *Oryzomys flavecens* (SABATTINI et al. 1977).

Recently it was shown that *Calomys callidus*, although not a demonstrated virus carrier in nature, could be persistently infected in the laboratory (VIDELA et al. 1985), thus providing yet another potential reservoir for Junín virus.

Crop management has been suggested as a means to flatten high rodent population density peaks, such as shifting to soya production in the most affected areas, which has been reported to favor rodent reservoir migration (KRAVETZ and DE VILLAFAÑE 1981; BUSH et al. 1984).

A seroepidemiologic survey showed a total prevalence of 12% infection in the endemic area, of which 8% were clinical cases with positive serology and 4% were residents without previous history of AHF, but with serum-neutralizing antibodies. These studies indicate that roughly one-third of human JV infection may course subclinically and that about 90% of the population in this zone is still susceptible to AHF (WEISSENBACHER et al. 1983a). During

the past years the steady expansion of the endemic area has raised the number of exposed people to 1.5 million (J.I. MAIZTEGUI, unpublished).

In 1983, residents in the south of Buenos Aires Province, an area not endemic for AHF, were serologically tested for anti-JV antibodies. Simultaneously virus and serum antibodies were sought in local wild rodents. Remarkably enough, findings were positive in two humans and two rodents (WEISSENBACHER et al. 1985a). This interesting finding indicates that, in addition to the present endemic area, other distant zones are potentially at risk for the appearance of AHF clinical cases.

The route of virus entry in human beings is mainly cutaneous and mucosal by means of skin abrasions, common in rural workers, or through conjunctival, oral, and/or respiratory mucosa by contaminated airborne dust during manual and mechanized work. Concomitant with mechanized work, numerous infected rodents are killed and their scattered remains contaminate the field. All these routes of viral entrance are confirmed by accidental laboratory infections when virus gains entry through airspray, pipetting, or the skin (WEISSENBACHER et al. 1976a, 1978a; WEISSENBACHER and DAMONTE 1983). Ready penetration by the mucosal route has also been demonstrated by oral or nasal infection of guinea pigs and marmosets (SAMOILOVICH et al. 1983a; 1984).

After penetrating through the skin or mucosa, the virus undergoes a 1 to 2-week incubation period, and initial symptoms are quite nonspecific. Among the first findings, and those most useful for reaching an early clinical diagnosis, are marked asthenia, muscular pain, dizziness, skin and mucosal rashes, lymph node enlargement, cutaneous petechiae, and retroocular pain. At 6–10 days after onset, symptomatology tends to worsen in most patients, when cardiovascular, digestive, renal, or neurologic involvement become more severe, together with hematologic and clotting alterations (MAIZTEGUI 1975; CINTORA 1977). Blood and urine tests show thrombocytopenia, leukopenia, albuminuria, and cylindruria (RUGIERO et al. 1964). In each individual case, findings are mainly hemorrhagic or neurologic and at 10–15 days, over 80% of the patients improve noticeably, while the remainder are prone to worsen. Total mortality reaches 16% in the absence of early convalescent plasma treatment (RUGGIERO et al. 1977). Mortality drops to 1%–2% when immune plasma is administered within 8 days of overt clinical disease (MAIZTEGUI et al. 1979). Regardless of the clinical form convalescence is quite lengthy, but total recovery takes place without sequelae, except for patients presenting the so-called late neurologic syndrome. Although different degrees of neurologic alterations are routinely observed in most cases of AHF, involvement is confined to the acute stage (MELCON and HERKOVITS 1981). However, 8%–10% of plasma-treated patients exhibit patent alterations in the central nervous system (CNS), such as ataxia, nystagmus, cerebellar tremors, and gait lateralization, which appear after recovery during frank convalescence (MAIZTEGUI et al. 1979). Recently evoked auditory potentials demonstrated lasting CNS impairment in most cases presenting with the late neurologic syndrome (CRISTIANO et al. 1985).

Clinical symptomatology induced by JV in humans closely resembles that reported for Machupo and Lassa viruses, ranging from inapparent infection

to lethal disease. However, in African hemorrhagic fever mortality is higher and person-to-person transmission frequent, unlike with AHF, where this is exceptional.

Reviews on several aspects of arenaviruses in general and JV in particular have been published with plentiful references to the literature then available. In the present review, we have attempted to update mainly the biological and biochemical properties of JV, the pathogenesis of AHF, and its treatment and prevention. Further data on other aspects of JV infection may be gleaned from previous reviews (METTLER 1969; COTO 1974; WEISSENBACHER et al. 1975; MAIZTEGUI 1975; WEISSENBACHER and DAMONTE 1983) and from references cited herein.

2 Properties of Junín Virus (JV)

2.1 Morphology and Chemical Composition

2.1.1 Morphology

JV particles had been seen in electron micrographs (EM) of thin sections of infected mouse embryo fibroblasts and suckling mouse brain before the arenavirus group was established (LASCANO and BERRÍA 1969). Typical arenavirus particles appeared in the extracellular space budding from the plasma membranes. Due to the virions' marked pleomorphism and peculiar aspect, it was only after Murphy's work (MURPHY et al. 1970; MURPHY and WHITFIELD 1975) that there was general agreement that these EM images actually depicted JV particles.

Like other arenaviruses, round, oval, or pleomorphic budding particles with a diameter of 50–300 nm were observed in ultrathin sections of JV-infected Vero cells (MURPHY and WHITFIELD 1975). The core exhibited a clear zone containing a variable number of granules, which resembled ribosomes. A dense peripheral zone made up the virus envelope covered with spikes was formed from the plasma membrane of the host cell by budding. It has been claimed that spike visualization is an artifact resulting from the depth of the tissue section (LASCANO and BERRÍA 1974; LASCANO et al. 1979). Studies with purified virus will help to clarify this point.

2.1.2 Genome

Initially, biochemical studies of JV were hampered by the lack of a suitable cell system to grow the virus in high titers. Later, BHK-21 cells infected at a moi of 3 LD_{50}/cell with MC2 strain, proved to be the best way to perform purification procedures (MARTÍNEZ SEGOVIA et al. 1974).

Nucleic acid isolation from purified virions and later analysis by PAGE have shown that the virus genome is composed of two large species of RNA, 33 and 25 *S*, with molecular weights of 2.4×10^6 and 1.34×10^6, respectively (AÑON et al. 1976). In addition, three small RNAs of 4, 5, and 5.5 *S* were

found along with 28 and 18 *S* RNAs, probably derived from cellular ribosomes. RNase sensitivity of all isolated RNAs proved their single-structure nature.

Presence of 18 and 28 *S* RNAs in purified virions grown with 0.1 μg actinomycin D, which otherwise inhibited more than 95% cellular rRNA, raised some doubts as to the origin of these RNAs (AÑON et al. 1976).

2.1.3 Polypeptide Composition

Six structural viral polypeptides, four of them glycosylated, were found in purified JV virions analyzed by SDS-PAGE (MARTÍNEZ SEGOVIA and DE MITRI 1977). Their estimated molecular weights were 91000 (VP1), 72000 (VP2), 64000 (VP3), 52000 (VP4), 38000 (VP5), and 25000 (VP6).

VP3 and VP5 were major components. VP1, VP2, VP4, and VP5 were glycosylated and later called G91, G72, G52, and G38. VP64 was assumed to be the nucleoprotein because it cosedimented with viral RNA (vRNA) in sucrose gradients, according to MARTÍNEZ SEGOVIA and DE MITRI (1977). These authors also reported that G38, the most prominent glycoprotein, is an envelope constituent because it can be removed with detergent from purified particles.

Protein analysis of purified virus under more stringent conditions has shown three structural polypeptides (GRAU et al. 1981): one nonglycosylated of 60000 and two glycosylated of 44000 and 39000–35000, respectively. Isolation of JV nucleocapsids by dissociation of virions with NP40 followed by centrifugation through a sucrose gradient also suggests that VP64 is the nucleoprotein.

Recent findings partly explain the discrepancy in the number of polypeptides reported from both groups. Analysis by immune precipitation and SDS-PAGE of intracellular [^{35}S]methiodine-labeled specific polypeptides demonstrated the synthesis of NP64 (previously named VP64) and two glycoproteins, G72 and G38 (DE MITRI and MARTÍNEZ SEGOVIA 1985). Pulse-chase experiments revealed that G72 is a precursor of G38, the latter presumably arising from proteolytic cleavage. No satisfactory explanation has been advanced for the presence in virions of G91, regularly found in electrophoretic profiles (DE MITRI and MARTÍNEZ SEGOVIA 1980), but it may be an aggregate of two or more polypeptides.

In addition to specific viral polypeptides, a protein of 43000 daltons has been identified by SDS-PAGE of purified JV (PASIAN et al. 1983). This polypeptide that comigrates with cellular actin, unlike other major specific viral polypeptides, was preferentially labeled in virus grown in prelabeled BHK-21 cells. Thus, by analogy with other enveloped viruses, it was postulated that actin was an integral part of the virion. Electrophoretic profiles of Triton-X-100 sodium deoxycholate fractions obtained from purified JV showed that only G38 was removed by the treatment, proving that actin is located within the virions (PASIAN et al. 1983).

2.2 Biological Activities of Virion Polypeptides

JV produces a soluble viral antigen (SAg) during its multiplication in BKH-21 cells (DE MITRI and MARTÍNEZ SEGOVIA 1980). SAg was extracted by ultracentri-

fugation from infected cells at 72–96 h postinfection after cell disruption. Supernatant fluid containing SAg had antigenic activity detectable by a complement fixation (CF) test and Ouchterlony double-diffusion test against JV hyperimmune serum.

To correlate viral polypeptides with their inherent antigenicity, purified virus radiolabeled with [^{3}H]amino acids and [^{14}C]glucosamine was disrupted by incubation with Triton X-100 and later centrifuged in a continuos sucrose gradient (CRESTA et al. 1980; DE MITRI and MARTÍNEZ SEGOVIA 1980). Polypeptide analysis by SDS-PAGE of the soluble fraction remaining at the top of the gradient showed that it contained only the major glycoprotein G38. Immunization of rabbits and guinea pigs with G38 emulsified with complete Freund's adjuvant elicited neutralizing antibodies against infectious virus. Furthermore, inoculated guinea pigs were protected against challenge with the pathogenic XJ strain of JV. The pellet fraction containing the other polypeptides (VF_1) was unable to induce neutralizing antibodies, but expressed CF activity (DE MITRI and MARTÍNEZ SEGOVIA 1980). VF_1 treated with RNase and centrifuged on a sucrose gradient again rendered a light fraction and material sedimented at the bottom of the tube (VF_2). CF activity was invariably associated with the denser fractions VF_1 and VF_2. Analysis of VF_1 and VF_2 by SDS-PAGE demonstrated that VF_1 contained NP64, GP72, and VP25. Pronase treatment of VF_2 generated a virus fraction VF_3 containing two nonglycosylated polypeptides of 20000 and 25000, similar in size to those obtained from SAg and exhibited CF activity, leading to the conclusion that NP64 is the soluble antigen.

2.3 Relationship to Other Arenaviruses

JV is a member of Tacaribe complex of the arenaviridae family. All members of the group serologically cross-react by CF (CASALS et al. 1975) and by the indirect fluorescent antibody (IFA) technique (WULFF et al. 1978), but are antigenically distinct by neutralization and immunoprecipitation tests (CHASTEL 1972b).

Tacaribe complex viruses cross-react strongly with each other but only weakly with the Old World arenaviruses lymphocytic choriomeningitis virus (LCMV), Lassa virus, and Mozambique virus (WULFF et al. 1978).

Despite the fact that LCMV and JV are antigenically distant, they can readily interact. Association of LCMV and JV was suspected because LCMV was isolated from *Mus musculus* captured in the endemic AHF area (SABATTINI et al. 1970). This natural association was confirmed by recent laboratory studies (REMORINI et al. 1985), which showed that nonhomologous reassortants were formed between LCMV and JV by mixed infection of cells with t_s mutants of both viruses. By cDNA hybridization and RNA fingerprinting, the formation of wild-type reassortants containing a genome formed with the *L* segment derived from LCMV parent, and the *S* from JV was demonstrated.

Biological and serological evidence indicates that the Tacaribe complex member most closely related to JV is Tacaribe itself, a relationship manifested in various ways.

It was reported that both viruses are indistinguishable using CF techniques because of a high degree of cross-reactivity (CHASTEL 1972a). Their close relationship demonstrated by means of polyclonal hyperimmune serum was confirmed recently with monoclonal antibodies raised against both viruses.

Five monoclonals prepared against Tacaribe virus cross-react with JV antigen (HOWARD et al. 1985). Reciprocally, all seven clones prepared against the XJC13 strain of JV gave positive reactions with Tacaribe-infected substrates and also recognized Machupo virus antigens. Since the antibodies raised against Tacaribe virus reacted with internal polypeptide N, it can be assumed that polypeptides belonging to Junín and Tacaribe viruses associated with nucleocapsids share several epitopes.

In contrast to JV, most members of the Tacaribe complex are nonpathogenic for guinea pigs, a differential property that served in exploring interrelationships by means of cross-protection tests (COTO et al. 1967; WEISSENBACHER et al. 1976b). Animals inoculated with a single Tacaribe virus dose were fully protected against JV challenge, confirming very early studies performed 20 years ago (PARODI and COTO 1964; TAURASO and SHELOKOV 1965). Three of ten guinea pigs inoculated with Machupo virus survived and became immune against JV (COTO et al. 1976). Pichinde and Tamiami viruses failed to protect guinea pigs, but a high survival was recorded for Amaparí virus-infected animals. Thus, Junín, Tacaribe, and Amaparí viruses appear closely related, in agreement with a previously reported serological analysis (CASALS et al. 1975). Interestingly, this virus grouping also arose from the response to cross-superinfection of Junín, Pichinde, Tamiami, Tacaribe, or Amaparí Vero-cell carrier cultures (DAMONTE et al. 1983). Specific cross-resistance was found among Junín, Tacaribe, and Amaparí. The origin of this immunity against Amaparí could not be explained but, for Junín and Tacaribe viruses, interfering articles (IPs) participate actively in this phenomenon.

Junín and Tacaribe viruses interact during mixed infection of RK-13 cells (DAMONTE and COTO 1985). When there was a 1- to 24-h delay between JV preinfection and Tacaribe virus superinfection, a variable increase in Tacaribe virus plaquing efficiency was observed. In virus progeny analyzed by neutralization, most particles formed were neutralized by antiserum against either JV or Tacaribe virus. These results indicate that during dual infection phenotypic mixing with JV or Tacaribe virus genomes enclosed within an envelope containing Tacaribe and JV glycoproteins had occurred.

The efficiency of poliovirus, Newcastle disease virus (NDV), measles virus, herpes simplex virus (HSV), Sindbis virus and vesicular stomatitis virus (VSV) was determined in Vero cells persistently infected with JV (DAMONTE et al. 1978a). VSV titers increased 5–15 times in JV-carrier cultures, while serological analysis of virus progeny showed the presence of pseudotypes formed with a VSV genome and JV envelope.

Although there was some evidence that JV could be neutralized by Tacaribe virus immune serum (HENDERSON and DOWNS 1965), doubts were raised whether specific neutralizing antibodies could account for protection observed in guinea pigs immunized with Tacaribe virus. Despite the fact that early protection could not be ascribed to heterologous neutralizing antibodies, (DAMONTE et al. 1978b),

6 weeks after Tacaribe infection the animals developed heterologous antibodies able to neutralize JV infectivity (WEISSENBACHER et al. 1976, 1977; COTO et al. 1980), and, following JV challenge, a typical secondary immune response was detected. Since in these experiments both viruses were replicated in suckling mouse brain, the specificity of these neutralizing antibodies was questionable, considering the possibility that they were raised against host-cell antigens. To clarify this point, guinea pigs were immunized with Tacaribe virus grown in RK-13 cells, Vero cells, or suckling mouse brain (COULOMBIE et al. 1984). Sera from animals bled at 60 days postinfection were assayed in neutralization tests against Tacaribe virus and JV grown in the same three substrates. Specific neutralizing antibodies against JV were found in all sera, ruling out the possibility that the antibodies were directed against cell antigens. However, it was observed that antibody titers were higher when neutralization was performed using immune serum prepared with virus grown in the host used for virus antigen, suggesting that during the process of budding viruses recruit cellular antigens that enhance the immune response.

Cross-reactivity between Tacaribe and Junín virus was recently studied at the molecular level (DAMONTE et al. 1985). By immune precipitation of [^{35}S]methionine-labeled JV or Tacaribe virus-infected cell lysates, all major virus-specific polypeptides from each virus were found to be reactive with not only homologous but also heterologous antisera.

This somewhat unexpected result prompted the analysis of cytosol preparations radiolabeled with [^{3}H]glucosamine of JV and Tacaribe virus by immunoprecipitation. As expected, the polypeptides displayed a higher degree of antigenic reactivity to the homologous than to the heterologous serum but, surprisingly, although JV antiserum failed to neutralize Tacaribe virus, it was still able to immunoprecipitate Tacaribe virus glycopeptides. This finding suggests that Tacaribe and JV share at least one antigenic determinant in their surface glycoprotein. The relationship between both viruses demonstrated by all available data justifies their inclusion in a separate subgroup in the Tacaribe complex.

A better understanding of JV interactions with related and unrelated viruses will be achieved by developing suitable molecular biology techniques. Although to date JV t_s mutants have been produced by FU mutagenization (CERIATTI et al. 1983, 1986), the genetic of this virus is still in its infancy.

3 Interaction of JV with Cells

3.1 Acute Infection

JV multiplies in a large variety of cells from different origins (COTO 1974), without apparent restriction at the entrance level. However, although abortive viral cycles have not been reported, replication is poor and courses without cytopathic effects.

Suitable plaque assays were developed in Vero cells (DAMONTE and COTO 1974) and in the human diploid cell line MRC-5 (WEBER et al. 1985), since the virus produced a characteristic cytopathic effect in both cell systems.

Host-cell interaction has been thoroughly studied in Vero cells infected with the XJ-Cl3 strain. Regularly, two types of particles are generated: plaque-forming units (PFU)- UV-sensitive cytopathic effect producers and IP (HELP 1980). The latter displayed an unusually high resistance to UV irradiation (HELP and COTO 1981; DAMONTE et al. 1983), but are completely neutralized by immune sera raised against infectious virus. To quantify IPs, an interference assay was developed based on their property of inhibiting all cytolytic activity of homologous virus by coinfection (HELP 1980). Interestingly, JV IPs also interfered with heterologous Tacaribe virus (HELP and COTO 1981; DAMONTE et al. 1983).

The synthesis of IPs is favored in cells infected at high moi or after serial passages of concentrated virus (HELP 1980). Virus cytopathogenicity for cells appears to be modulated by IPs. Thus, it is a typical feature of JV titration in Vero cells that monolayers inoculated at high moi appear much like normal cells and the there is no direct correlation between expected PFU according to virus inoculum and counted PFU. IPs are unable to replicate by themselves; it was observed that after three successive passages in cells, viral infectivity dropped 5 logs, whereas interfering activity peaked to a maximum. After that, a cyclic-interdependent pattern of both standard virus production and IP synthesis was found to develop (HELP and COTO 1981). A dose-response curve of IP activity indicated that one IP was sufficient to inhibit standard virus mulipli-cation in the same cell (HELP and COTO 1981). IPs can be concentrated by ultracentrifugation at 100000 *g*, but they are very difficult to separate from standard virus in sucrose gradients (G. HELP, personal communication). Generation of IPs is not only a feature of in vitro infection, but also occurs in the brains of newborn mice infected with JV (HELP and COTO 1980). The presence of IPs was evident when it was observed that the deaths of animals inoculated with concentrated virus were delayed in comparison with those of mice infected with lower virus doses. By passing serially concentrated virus through newborn mouse brain, a decrease in viral infectivity and a concomitant increase of interfering activity was observed. Thus IP generation seems to be a regular event in JV replication, which is dependent on the ratio of IPs to standard virus in inoculum. The possible use of IPs as antigen for vaccination or whether they play a role in viral persistence in cricetids, which are the JV natural hosts are matters of speculation.

3.1.1 The Replicative Cycle

Knowledge of the events of the JV replication cycle is still fragmentary. A serious drawback to tracing the fate of parental virus and the appearance of new virus genomes is the lack of inhibition of host-cell protein synthesis. Under one-step conditions, virus progeny in Vero cells is detected in the extracellular fluid at 10–12 h postinfection (COTO and DE VOMBERGAR 1969). Only 3% of the infected cells are virus yielders, as determined by infectious center assay (COTO et al. 1970), in agreement with the percentage of cells synthesizing cytoplasmic antigen visible by immunofluorescence (IF). The number of infected cells increased with time and 72 h postinfection a maximum of 53% of infectious centers was detected. Under infection conditions of 1 PFU/cell, to avoid IP

interference, maximum titers were attained at approximately 40 h postinfection. At that time there was no marked cytopathic effect, which later fully developed at 72–96 h postinfection. Due to the difficulty of infecting all cells, any attempts at biochemical characterization early after infection failed. At 48 h postinfection, when several virus cycles had occurred, specific viral polypeptides could be detected by immune precipitation of [^{35}S]methionine-labeled, JV-infected, Vero cell lysates. A glycoprotein precursor (GPC) of NP64 and virion glycopeptides G38 and G52 were revealed by SDS-PAGE (DAMONTE et al. 1985).

Although BHK cells are better virus yielders, the virus growth cycle is similar to that described for Vero cells. In the former analysis, immunoprecipitation and SDS-PAGE of intracellular [^{35}S]methionine-labeled specific JV virus polypeptides demonstrated the synthesis of NP64 from 24 to 96 h postinfection. At 48 h, G72 and G38 appeared, but, as mentioned before, while G72 decreased, G38 synthesis was maintained throughout the 96-h observation period (DE MITRI and MARTÍNEZ SEGOVIA 1985).

3.1.2 Virus Entrance and Intracellular Events

It is not known how JV penetrates cells, but indirect evidence that a receptor-mediated endocytosis mechanism is operating can be inferred from experiments with amantadine-HCl. It was reported that JV replication is inhibited in the presence of this compound (COTO et al. 1969), which is now considered a lysosomotropic agent. Colchicine and NH_4Cl, at nontoxic concentrations for cells, also inhibit JV replication (C.E. COTO, to be published), lending further support to the participation of endocytosis in JV-cell entrance.

The presence of an RNA-dependent RNA polymerase in arenaviruses has been reported only for Pichinde virus (CARTER et al. 1974; LEUNG et al 1979). RNA polymerase activity was found in partially purified virions of JV grown in Vero cells (MERSICH 1980). For optimal enzyme functioning several requirement were essential. Significant amounts of [^{3}H]UTP were incorporated into acid precipitable protein at 32° C only if virus was treated with NP40. For maximum activity the presence of divalent cations Mg^{2+} and Mn^{2+} was needed. Substitution of Mn^{2+} by Na^{+} failed to alter the incorporation observed. Omission of CTP anulled the reaction, but omission of GTP reduced incorporation by 90%. The presence of an NTPase activity in all preparations, probably derived from cellular membrane contaminants, hindered all polymerase activity determinations. There are no reports on the presence of RNA-dependent RNA polymerase activitiy in infected cell. The appearance of a 200000 dalton polypeptide in BHK cells infected with JV, immunoprecipitable by antisera specific for the nucleoprotein, led to the assumption that this internal protein could represent RNA polymerase (DE MITRI and MARTÍNEZ SEGOVIA 1985).

A dependence of JV replication on the integrity of nuclear function was suggested by the demonstration that JV multiplication was severely arrested in UV-irradiated cells (COTO and HELP 1971). On the other hand, inhibitors of DNA synthesis, like IUdr, 5-BrUdr, or ARA C (COTO and DE VOMBERGAR 1969; MARTÍNEZ SEGOVIA and GRAZIOLI 1969; COTO and HELP 1971) had no

effect on JV replication. Inasmuch as low concentrations of actinomycin D allowed normal viral yields (COTO and DE VOMBERGAR 1969), the question of which cellular function was essential was raised.

The finding that JV multiplication was inhibited in cells incubated with 10 μg/ml of α-amanitine (MERSICH et al. 1981) led to the assumption that cellular DNA-dependent RNA polymerase II activity was needed for virus replication. Activities of cell nuclear polymerases were investigated in Vero cells infected with the XJCl3 strain of JV. It was found that isolated nuclei showed an increased RNA polymerase II activity, which peaked at 6 h postinfection (MERSICH et al. 1981) and declined to normal levels at the time when virus progeny appeared in cell supernatants and RNA polymerase I was inhibited. These results strongly suggest that JV replication requires the synthesis of a cellular mRNA whose function is still unknown.

3.1.3 Morphogenesis

Like other enveloped viruses JV is inhibited by the glucose derivatives glucosamine (2-amino-D-glucose) and 2-deoxy-D-glucose (2 DG) (LEON et al. 1976). The effects of both inhibitors on virus multiplication were studied in Vero cells by adding each compound immediately after infection. In the presence of 2 DG at a concentration of 20 m*M*, the yield of extracellular virus, at 48 h postinfection, was reduced 2 logs while glucosamine at a concentration of 16 m*M*, completely blocked virus production.

Glucosamine failed to affect virus adsorption or penetration (LEON et al. 1977). Inhibition of JV replication by glucosamine was fully reversible (LEON 1980). Virus sythesis was arrested late in the replication cycle, since drug removal and further incubation in fresh medium, free of inhibitor, allowed the delivery of infectious virus to the supernatant 4–5 h later. The release of JV particles was normally regained even when cells were treated for as long as 72 h (LEON and COTO 1980).

Immunofluorescent studies coupled with electron microscopy revealed that when Vero cells infected with JV were cultured in the presence of glucosamine, no infectious virus, surface antigen, or budding particles were found (MARTÍNEZ PERALTA et al. 1979a). In contrast, the percentage of cells showing cytoplasmic antigen was similar in glucosamine-treated and untreated cultures. From these results, the conclusion was drawn that glucosamine specifically affects virus replication through the inhibition of viral glycoprotein synthesis.

Further support for this assumption came from experiments performed with [^{3}H]uridine-labeled virus grown in glucosamine-treated or untreated cultures (DAELLI and COTO 1983). Cell culture fluids were centrifuged 2 h at 100000 *g* and the suspended pellets further purified in a sucrose gradient. Virus from untreated cultures banded at a density expected for JV (AÑON et al. 1976). However, no peak was observed in the radioactive profile of the sucrose gradient loaded with virus purified from glucosamine-treated cells, indicating that no particles were delivered to the supernatant.

Another inhibitor of glycoprotein synthesis, the antibiotic tunicamycin, also inhibited JV multiplication (DAELLI and COTO 1982, 1983; PADULA and DE MARTÍNEZ SEGOVIA 1984).

Studies performed in Vero cells showed that noninfectious virus formed if 1 μg/ml tunicamycin was added immediately after virus adsorption and remained to the end of the replicative cycle. Addition of the antibiotic at 24, 48, or 72 h postinfection immediately arrested virus synthesis. Unlike glucosamine, tunicamycin had an irreversible effect (DAELLI and COTO 1983).

Virus budding occurs normally as demonstrated by sucrose gradient analysis of purified virus grown in the presence of tunicamycin (DAELLI and COTO 1983).

PADULA and DE MARTÍNEZ SEGOVIA (1984) described the effect of tunicamycin on JV replication in BKH-21 cells. Sucrose gradient analysis of purified virus labeled with [^{35}S]methionine showed that virus-infected, treated cells released 70% as many virus particles as control cultures, although these were noninfectious. They also found by SDS-PAGE analysis of purified [^{14}C]glucosamine-labeled virus that budding particles contained normal amounts of the polypeptides NP64, G55, and G72, but were deprived of glycopeptide G38; this indicates that the presence of carbohydrates is important for viral infectivity, but plays no role in the transport of viral proteins through cellular membranes.

In summary, the differential effects of glucosamine and tunicamycin on JV replication clearly show that inhibition of glycosylation fails to affect intracellular transport of viral polypeptides, its insertion in the cellular membrane, or its extrusion from the cell. Therefore, glucosamine should not interfere with cellular processes responsible for viral protein transport, other than glycosylation.

3.2 Persistent Infection

Characteristically, after a period of high virus production, not necessarily followed by cytopathic effects, as in the case of BHK-21 cells (CARBALLAL et al. 1980), JV establishes a persistent lifelong infection.

Vero-cell carrier cultures are morphologically indistinguishable from normal, uninfected cells and have similar growth characteristics (BOXACA 1970; DAMONTE and COTO 1979; COTO et al. 1979). Persistently infected (PI) Vero cells were not cured by serial cultivation in media containing JV-neutralizing antibodies (BOXACA and GIOVANNIELLO 1977). About 20% of cells contained cytoplasmic JV antigens, but only 4% showed cell-associated antigen (COTO et al. 1979), although by infectious center assay, virus producer cells were undetectable (CANDURRA and DAMONTE 1985). Thus, JV-PI Vero-cell cultures consist of a minority of cells releasing virus, cell containing only cytoplasmic antigen, and cells with no viral antigen expression. This seems a puzzling observation since PI-Vero cells are fully refractory to homologous superinfection (BOXACA 1970; COTO et al. 1979; DAMONTE et al. 1983), but it may provide a key to explain persistence at the cellular level.

Independent studies of PI-Vero cells have shown that in cells maintained by weekly serial transfers over a period of 100 days or more, virus released into the supernatant followed a cyclic pattern (COTO et al. 1979; DAMONTE and COTO 1979; CANDURRA and DAMONTE 1985), with long periods of virus disappearance.

It appears that JV undergoes a normal replicative cycle in cells showing positive membrane IF without killing them. The small amount of virus released

cannot be amplified by successive growth cycles due to the superinfection resistance of remaining cells. Therefore, long nonvirogenic periods occur until there are enough susceptible cells to allow virus multiplication. This explanation of how persistent infection is regulated somehow fits the model of transient infection proposed by Hotchin for LCMV (HOTCHIN 1974). However, other reported observations on PI-Vero cell cultures should be considered before a theory to explain virus persistence may be advanced.

Virus mutants with a t_s phenotype regularly emerged in PI-Vero cells (DAMONTE and COTO 1979; COTO et al. 1981). Mutants with altered pathogenicity for guinea pigs were also found (BOXACA and GIOVANNIELLO 1977). Moreover, several kinds of mutants (plaque morphology, t_s, and lower cytopathogenicity for newborn mice) were isolated from supernatants of JV PI-MRC-5 cells (WEBER et al. 1985), a carrier culture whose properties closely resemble those of PI-Vero cells.

Besides the detection of virus variants, the presence of interfering particles in PI-Vero cells has been repeatedly reported (HELP et al. 1976; COTO et al. 1977; DAMONTE et al. 1983; CANDURRA and DAMONTE 1985). IP detection requires concentrating cell supernatants 100-fould by pelleting at 100000 g; this indicates that unlike PFU viruses, IP synthesis is reduced. Since IPs interfered with the synthesis of regular PFU particles, it can be speculated that IPs play a major role in maintaining virus persistence.

However, the overall picture is more complicated. A few studies suggest that host cells may regulate the infection. Treatment of JV-infected Vero-cell carrier cultures with actinomycin D or cycloheximide increased the number of cells with positive IF staining of surface viral antigens and induced the appearance of plaquing virus (COTO et al. 1977). This finding indicated that a cellular protein product was required to maintain the virus in a repressed state.

Recently, the interplay of infectious virus production, synthesis of IP, and presence of t_s mutants was studied during 110 days in PI-Vero cells subjected to different culture conditions (CANDURRA and DAMONTE 1985). Carrier cultures maintained in stationary conditions continuously released PFU viruses, whereas PI-Vero cells passaged on a regular schedule, at 3- or 7-day intervals exhibited a cyclic pattern tending toward rapid PFU disappearance. The production of IP in stationary cultures was delayed and lower than in actively growing cells where the metabolic state failed to affect the emergence of t_s mutants.

Considering all available data, it is not easy to visualize the mechanism of JV persistence in cultured cells at the molecular level. Although IPs may actually regulate production of standard virus, their own synthesis requires not only the presence of standard virus (acting as a helper), but also the expression of a host-cell product.

4 Chronic Infection in Animals

4.1 Calomys musculinus

Studies performed during the 1960s demonstrated that the field mouse *C. musculinus* was the main JV reservoir. In the endemic area as many as 21% of captured

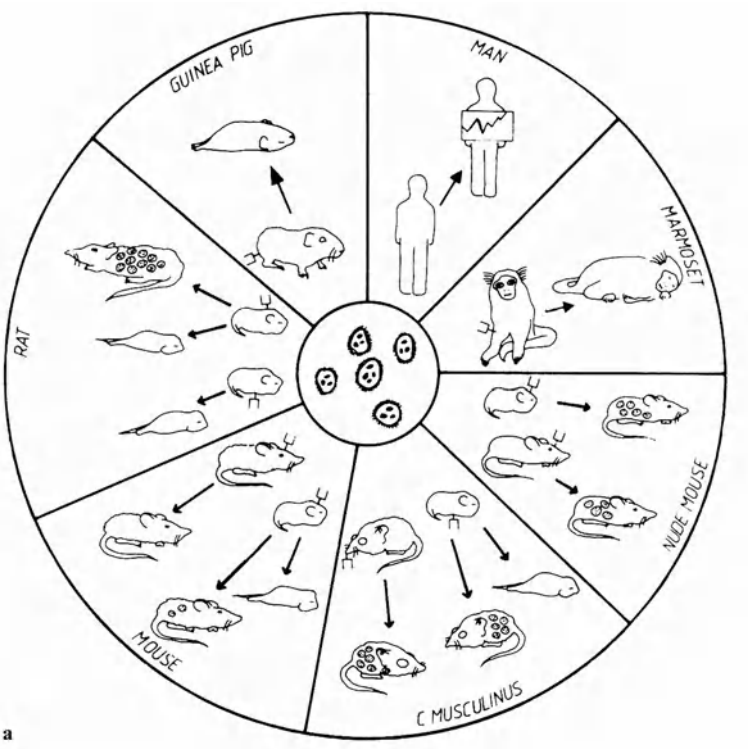

Fig. 1a, b. Patterns of the evolution of infection in man and other species with the pathogenic XJ (**a**) and the attenuated XJCl3 (**b**) strains of Junín virus. The outcome of the disease and its eventual persistence seem to depend on numerous factors, e.g., virus strain, inoculation route, age at the moment of infection, immunological status, and animal species (see text Sects. 4, 5, and 6 for details)

rodents were infected, as shown by positive JV isolation from blood and viscera or by the demonstration of anti-JV complement-fixing antibodies in serum. (SABATTINI et al. 1977). Other wild rodents were also found harboring the virus, such as *Calomys laucha* and *Akodon azarae*, but the number of infected animals was lower than for *C. musculinus* (SABATTINI and CONTIGIANI 1982). Laboratory studies demonstrated that infection of *C. musculinus* was mainly horizontal, by means of close contact between animals, and that saliva was the main infective secretion, although urine could also be contaminated. The maximal viral concentration was found in the salivary glands with shedding of a large number of viral particles into the ducts (MARTÍNEZ PERALTA et al. 1979b). Viremia as well as anti-JV antibodies were present temporarily. Although virus was also found in other organs, especially lymph nodes and spleen, the fact that virus production was higher in the salivary glands supported the opinion that saliva was the main route for natural virus spread. No congenital infection was noticed, but newborn rodents became rapidly infected when nursed by infected mothers (SABATTINI et al. 1977).

Studies during the past few years have shown that persistent arenavirus infection in the natural hosts may no invariably course in subclinical fashion.

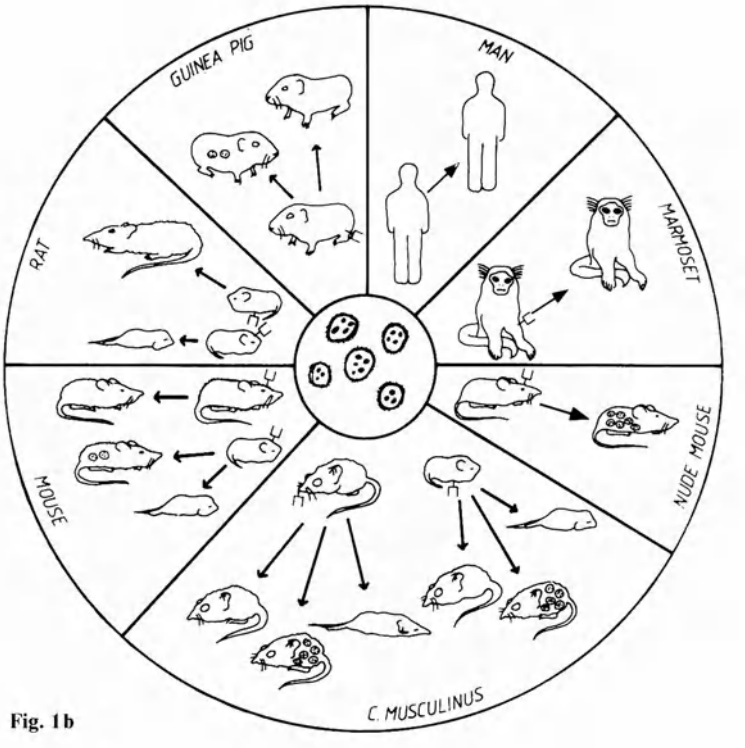

Fig. 1b

To illustrate, *C. musculinus* infected with wild strains of JV present an increase in late mortality; their growth is retarded and fertility decreases (VITULLO et al. 1985). Newborn *C. musculinus* infected by the intraperitoneal route with the XJC13 attenuated strain proved to be susceptible, since 50% of these animals died between 14 and 28 days post infection (LAMPURI et al. 1982; LAGUENS et al. 1982a). The virus was selectively neurotropic, rarely caused viremia, and occasionally disseminated to other viscerae. Susceptibility decreased with age, and animals older than 20 days were resistant when infected intraperitoneally. However, adult rodents could be infected by the intranasal route and developed a neurological illness with 50% mortality (LAGUENS et al. 1982b). Surviving *C. musculinus*, infected either perinatally or as adults, followed three different patterns (Fig. 1). Some animals presented to evidence of disease, some recovered after developing a clinical neurologic disease, and the remainder manifested persisting neurologic symptoms associated with growth arrest. This last group showed marked meningoencephalitis with high concentration of virus in the brain and JV antigens in cortical neurons, cerebellar granular, and Purkinje's cells. In animals belonging to the first two groups, no virus could be isolated from the brain or other organs, and in the CNS no antigens or lesions could be demonstrated. In all groups neutralizing anti-JV antibodies were found with no difference between persistently infected animals and those that cleared the virus.

This persistent infection in a natural reservoir, leading to marked pathological alterations, is unique for arenaviruses since lesions associated with a persistent infection, if any, are subtler and fail to show inflammatory infiltrates or tissue destruction. The pathogenesis of tissue damage in *C. musculinus* infected with the XJCl3 strain is probably immunologic, at least for the acute illness. Death and incidence of disease can be significantly reduced by administration of antithymocyte serum to newborn animals (COULOMBIÉ et al. 1983). In this model, fetuses from chronically infected mothers were free from infection, confirming findings with wild JV strains. Interestingly, a reverse path of infection was observed as mothers from experimentally infected litters also became infected by an undetermined route (LAMPURI et al. 1985). These studies, although performed with a laboratory strain, suggest that in nature there exist viral strains pathogenic for the normal reservoir. In support, several strains obtained from captured wild rodents lacked pathogenic effects similar to the XJCl3 strain (CONTIGIANI and SABATTINI 1986).

The mechanisms leading to viral persistence in *Calomys* are still almost unexplored, due in part to the lack of inbred strains, which prevents adequate immunologic studies. Up to now, persistent infection has been correlated with high titers of anti-JV neutralizing antibodies. Administration of cyclophosphamide in doses interfering with antibody synthesis failed to modify the course of the infection in neonates and adult *C. musculinus* (COULOMBIÉ and COTO 1985). Interestingly, virus recovered from brains of chronically infected animals was neutralized by serum from the same animal which completely neutralized the viral stock employed for infection (LAMPURI et al. 1985). Serological variants of JV were observed in blood of chronically infected *C. musculinus*, suggesting that this may be one of the mechanisms leading to persistence (ALCHE and COTO 1986).

Experimental infection of newborn *C. musculinus* with the XJ strain leads to a lethal disease in most cases, although survivors develop persistent infection. In adult animals infected with the same strain by the intranasal route, mortality does not follow but most develop the persistent infection (M.S. MERANI and M.A. CALELLO, unpublished).

4.2 Mouse

The newborn mouse is highly susceptible to any strain of JV administered by any route (NOTA et al. 1977). Animals develop an immune cell-mediated lethal meningoencephalitis (SCHMUÑIS et al. 1967). Recently it has been observed that the few survivors develop a persistent infection (Fig. 1), and virus can be recovered from the brain when they exhibit mild meningoencephalitis (RABINOVICH et al. 1984). Thymectomy, antithymocyte serum, and cyclophosphamide prevent the development of the disease (WEISSENBACHER and DAMONTE 1983). A similar situation occurs in congenitally athymic nu/nu mice, which develop a persistent infection with no tissue damage (WEISSENBACHER et al. 1983a, b). Reconstitution of infected thymectomized mice with immune splenocytes leads to a neurologic lethal disease (WEISSENBACHER et al. 1969a) confirming the immune pathogenesis

of tissue damage. In normal mice susceptibility to the virus decreases with age and, after weaning, animals are resistant. Infected adult mice develop neutralizing antibodies, and virus is rapidly cleared. Immune depression induced by treatment with cyclophosphamide or antithymocyte serum renders adult mice susceptible to JV (NEJAMKIS et al. 1980) indicating that resistance is dependent on the ability to develop an immune response. Curiously, the opposite is seen when adult nu/nu athymic mice are infected (WEISSENBACHER et al. 1986a). Animals show a persistent infection with high virus yield, no antibodies, and no pathological alterations. These results, although contradictory, point out that in the mouse persistent infection depends on immunological alterations, mainly of the T-cell compartment. In support, it was observed that more mice survived with persistent infection when inoculated within 24 h of birth. Besides, JV replicated earlier and in higher titers in the thymus and bone marrow than when inoculation was carried out at 2–3 days of age (CALELLO et al. 1985).

4.3 Rat

The adult rat is resistant to JV, but newborn and suckling animals differ in their responses according to the viral strain and route of inoculation (AVILA et al. 1981a). In rats under 3 days of age, the XJ strain inoculated by the intracerebral route produces a mild neurologic disease with low mortality. Animals recover and develop high titers of neutralizing antibodies, although some present with arrested growth and neurologic symptoms. Examination as late as 24 months after infection shows brain and cerebellar atrophy with JV antigens present in most cortical and Purkinje's cells and budding virions (LAGUENS et al. 1983a, WEISSENBACHER et al. 1986b). Virus usually cannot be isolated, probably because of the high levels of neutralizing antibodies. The exception is that infective virus can be isolated by cocultivation with permissive cells. Intracerebral infection of newborn rats with the XJC13 strain leads to an invariably fatal neurologic disease. The newborn rat is thus a biological marker for the virulence of JV strains (AVILA et al. 1981a). These differing responses to viral strains are lost in 10-day-old rats, which are susceptible to both strains. The age-dependent susceptibility associated with the virus strain, also observed in the 14-day-old mouse, which is more susceptible than newborn animals for some JV strains (CONTIGIANI and SABATTINI 1977), poses the interesting but as yet unsolved possibility of different mechanisms of tissue damage. Although in newborn mice and rats a T-cell-dependent immune mechanism appears to be involved, in older animals other mechanisms such as cytotoxicity by natural killer cells remains to be explored.

4.4 Primates

The marmoset *Callithrix jacchus* develops an acute and lethal disease when inoculated with the pathogenic JV strain. A different situation emerges when death is prevented with anti-JV serum (AVILA et al. 1986). One-third of surviving animals present neurological symptoms, and in one animal, persistence of virus

associated with transversal myelitis was demonstrated as late as 90 days after infection. The relevance of this finding to the so-called late neurological syndrome appearing in human AHF after treatment with immune plasma will be considered later. The South American primate *Cebus sp.* is partly resistant to JV, although transient disease can be observed (CARBALLAL et al. 1983). However, pathologic studies of the brains in infected animals showed a mild meningoencephalitis as late as 90 days after infection. Although apparently this lesion was self-limiting and the animals recovered, there is a possibility of prolonged infection.

5 Pathogenesis of AHF

5.1 Pathology of Human AHF

In spite of the many years that have elapsed since the first recognition of AHF in man, few studies are available describing pathologic features in fatal cases. Most lesions are nonspecific and consist of a generalized vasocongestion with multiple hemorrhages in the gastrointestinal mucosa, different organs, and subcutaneous tissue (ELSNER 1977; GALLARDO 1970). In half the cases the lungs show evidence of secondary bacterial infection (lobar and lobular pneumonia and pulmonary abscesses). The kidneys show tubular and papillar necrosis and in the liver scattered necrotic hepatocytes are usually observed. Massive liver necrosis is exceptional. In the CNS no conspicuous alterations are present, in spite of the appearance of neurologic symptoms in most cases. A mild meningitis and perivascular cuffing with mononuclear cells in the brain, have been reported in 3 of 14 cases. The absence of definite CNS pathologic alterations was confirmed by further studies (LAGUENS et al. 1977; MAIZTEGUI et al. 1975). The only lesions consistently found in fatal cases are present in the lymphatic tissue and bone marrow. The latter shows a global cell depletion with an even more marked decrease in the number of erythroblasts. This observation was confirmed by in vivo studies of bone marrow aspirates and biopsies (PONZINIBBIO et al. 1979). The lymphatic tissue has widespread necrosis, especially in the splenic white pulp and cortical and paracortical areas of the lymph nodes (GONZÁLEZ et al. 1980). These lesions are not disseminated and some lymph nodes appear hyperplastic with erythrophagocytosis. At postmortem, the highest virus titers are found in the spleen, lymph nodes, and lungs (GONZÁLEZ et al. 1980). Occasionally in the liver and bone marrow virus levels are higher than those present in the peripheral blood. Attempts to isolate virus by inoculating newborn mice with CNS structures (brain, pons, peduncles, cerebellum, and corpus striatum) yield negative results (R.P. LAGUENS, unpublished results). IF demonstrates JV antigen in cells presumably belonging to the mononuclear-phagocytic system in the lymphatic tissue, lung, and liver (LAGUENS et al. 1977; MAIZTEGUI et al. 1975). In the different parenchyma (hepatocytes, renal tubes, glomerular epithelial cells, and pancreatic acini) JV antigens can only occasionally be demonstrated (COSSIO et al. 1975; GONZÁLEZ et al. 1978). In the CNS the capillary

endothelium shows viral antigens apparently complexed or associated with autologous IgA, IgG, and IgM (Cossio et al. 1977).

5.2 Pathology of Experimental Infection

As adults few animal species are susceptible to JV infection. However, the guinea pig and marmoset (*C. jacchus*) develop a disease sharing many clinical and pathological features with the human illness.

Guinea pigs infected with the XJ strain develop a lethal disease lasting 10–20 days according to the amount of virus employed for infection (Guerrero et al. 1977; Elsner et al. 1976). All animals have pronounced leukopenia and thrombocytopenia, and necropsy shows massive necrosis of bone marrow and lymphatic tissue and interestitial pneumonia. Multiple hemorrhages are present in the lungs, adrenals, omentum, gastrointestinal mucosa, and subcutaneous tissue. Sequential studies have shown that the interestitial pneumonia is similar to the human adult pulmonary distress syndrome and that in early stages a massive polymorphonuclear leukocyte infiltrate is present in the alveolar capillary lumina (Cossio et al. 1983). In the CNS no alterations are found. All animals show viremia, and the highest virus yields are obtained from bone marrow, spleen, lymph node, and lungs; no virus can be recovered from the CNS when animals are inoculated by peripheral routes (Oubiña et al. 1984a). Virus can be excreted in urine and saliva from the 7th day postinfection (Guerrero et al. 1977). Infection of guinea pigs with other JV strains produces a different clinical and pathological picture. XJC13, usually considered attenuated for this species, induced 16% mortality in a controlled study with a large number of animals (Avila et al. 1981b). Sequential studies indicated that infection with this strain produced a constantly self-limiting meningoencephalitis and pancreatitis, but spared the lymphohemopoietic tissues (Laguens et al. 1983b). Virus replication was discrete and transient, with only sporadic viremia, but viral antigens were present in cortical neurons and capillary endothelia. Infection with another strain (MC2) of intermediate pathogenicity between XJ and XJC13 produces a lethal disease in about 70% of animals. In addition to meningoencephalitis, involvement of the lymphohemopietic tissue is observed, and virus titers in organs and viremia are higher than caused by infection with the XJC13 strain (Avila et al. 1981b), suggesting that strains of JV differ in tissue tropisms. The more pathogenic strains such as XJ are viscerotropic, mainly lymphotropic, and the more attenuated ones are mainly neurotropic.

Strains of intermediate pathogenicity are both lympho- and neurotropic. However, in a deeper analysis it appears that any strain of JV is neurotropic of guinea pigs, and that neurotropism becomes apparent in animals living long enough to allow virus to reach the CNS. When death is delayed by treatment with immune serum, guinea pigs infected with the XJ strain develop encephalitis, and virus can be recovered from the brain (Laguens et al. 1983b). The factors that determine virus replication in the brain remain to be ascertained. An enhanced neurotropism has been obtained after serial passages in guinea pigs of a pathogenic JV strain (Boxaca et al. 1984a), suggesting selection of a viral

subpopulation. The presence of anti-JV antibodies also seems to play some role, since with attenuated strains the CNS becomes involved when anti-JV antibody synthesis occurs, and with pathogenic strains, passively administered antibodies lead to the same outcome.

In a systematic study of sensitivity of South American primates it was found that the marmoset *C. jacchus* was susceptible to JV, developing a fatal disease when inoculated with the XJ strain (WEISSENBACHER et al. 1979). Other species, i.e., *Alouatta caraya* (WEISSENBACHER et al. 1978b), *Saimiri sciureus* (FRIGERIO et al. 1982), and *Aotus trivirgatus* (SAMOILOVICH et al. 1983b) proved to be resistant, although these animals developed antibodies after inoculation, indicating an inapparent infection.

Infection of *C. jacchus* with the XJ strain leads to an invariably fatal disease between the 3rd and 4th week postinfection (WEISSENBACHER et al. 1979). Infected animals develop anemia, leukopenia, and thrombocytopenia and, in all cases, multiple hemorrhages and severe meningoencephalitis with extensive brain necrosis and demyelinization (GONZÁLEZ et al. 1983). The remaining viscerae show no significant alterations. The lymph nodes appear hyperplastic, and erythroblastopenia is present in the bone marrow. IF shows JV antigenic determinants in neurons and blood vessels and in cells resembling macrophages in the lymphatic tissue and lungs. However, virus can be recovered from blood, brain, bone marrow, and spleen in high titers.

5.3 Interaction of JV with Antigen-Presenting Cells

IF of lymphatic tissue and viscerae of man and animals infected with JV show that most antigens are located in cells morphologically akin to macrophages (GONZÁLEZ et al. 1980; OUBIÑA et al. 1984b). These cells, isolated either from human peripheral blood, or mouse or guinea pig spleen or peritoneum, are efficient substrates for JV replication (GONZÁLEZ and LAGUENS 1981; GONZÁLEZ et al. 1982). Virus yield are high although only about one-third of cells appear to be infected, as shown by IF. Sequential studies reveal cyclic viral production, suggesting a persistent infection. Apparently cells are not damaged during infection with attenuated or pathogenic viral strains and display normal enzyme and phagocytic activities. JV replicates actively *in vitro* in peritoneal macrophages from newborn and adult *C. musculinus* (COULOMBIÉ et al. 1986). However, concerning infection in vivo by the intraperitoneal route, virus is associated with peritoneal macrophages from neonates, but not from adults. This could partly explain the resistance of adults to infection with JV by the intraperitoneal route (LAMPURI et al. 1982). Recently it has been shown that guinea pig splenic dendritic cells are the first targets to be associated in vivo with JV (LAGUENS et al. 1983c). With pathogenic strains the virus is found later to replicate in macrophages, a fact not observed with attenuated strains. The mechanisms that prevent replication of attenuated strains in macrophages remain to be elucidated. Production of interferon does not seem responsible for differential cell tropism, since infection in vitro with both pathogenic and attenuated strains induces splenic dendritic cells to produce interferon (LAGUENS et al. 1986a).

5.4 Polymorphonuclear Leukocytes and AHF

Humans and adult susceptible animals infected with JV show a severe neutropenia, whose origin is poorly understood. In man the bone marrow is markedly erythroblastopenic, but the remaining cells belonging to the granulocyte and megakaryocyte series are relatively well preserved.

In guinea pigs the neutropenia precedes by several days the onset of bone marrow necrosis. Electron microscopy and IF studies have shown that in the bone marrow JV is associated with macrophages and megakaryocytes (CARBALLAL et al. 1981a, b) but not with components belonging to the granulocyte lineage. The increase in plasma enzyme levels of polymorphonuclear (PMN) origin (KIERZENBAUM et al. 1970) led to the assumption that cell destruction rather than impaired generation or differentiation of PMN leukocytes could be responsible for neutropenia. Recently it has been shown that human PMN leukocytes can be damaged directly by JV in an in vitro system (LAGUENS et al. 1986b, c). Incubation of PMN cells with JV provoked a rapid increase in the elimination of lysosomal and cytoplasmic enzymes and death of more than 50% of cells in a 10-h assay. Specific neutralization of virus prevented the effect as well as inactivation with UV. The mechanisms leading to cell damage remain to be determined. It has been shown that stabilizing cell membrane drugs, such as lidocaine, prevent viral action. Cytochalasin B or colchicine have no effect, suggesting that lysosomes do not participate in cell injury (PONZINIBBIO et al. 1982). The progress of JV infection in guinea pigs with extreme neutropenia induced by specific anti-PMN serum was more severe than in untreated animals (P.H. GONZÁLEZ, unpublished). Guinea pigs died earlier, and higher virus yields were obtained in their organs. Pathologic studies showed similar lesions in untreated and treated animals with the exception of the lung, which was normal in guinea pigs receiving anti-PMN serum. These observations suggested that in JV infection PMN leukocytes may play a defensive role but in addition they could also induce or amplify lung damage.

5.5 Immune Response

The human disease is marked by immune depression. One of the characteristics of the illness is the lack of demonstrable antiviral antibodies. Only at 1–3 weeks after the onset of symptoms can anti-JV antibodies be shown by IF, neutralizing antibodies developing later (DAMILANO et al. 1983). Secondary severe infections are relatively frequent, and pneumonia, bronchopneumonia, and pulmonar abscesses were found in 7 of 14 necropsies (ELSNER 1977).

Preliminary studies showed a decrease in the number of peripheral blood lymphocytes with the ability to form E and EAC rosettes (ARANA et al. 1977). Recently a significant decrease in the number of $OKT4^+$ peripheral blood lymphocytes was reported, with normal numbers of cytotoxic-suppressor lymphocytes, indicating selective lymphocyte subpopulation damage. This situation is transient, and in early convalescence the number of circulating lymphocytes, and the $OKT4^+/T8^+$ relationship returns to normal levels. (VALLEJOS et al. 1985).

In guinea pigs infected with the XJ strain, primary and secondary immune responses to sheep red blood cells are markedly depressed (PARODI et al. 1967, 1970). Cell-mediated immunity is also depressed, as shown by skin tuberculin reactivity (CARBALLAL et al. 1981 b). Lymphatic tissue destruction has been held to be the main cause of the generalized immune depression.

5.6 Pathophysiology

The pathogenesis of tissue damage in AHF remains speculative. Although a T-cell-mediated immune mechanism has been demonstrated for perinatal infection in mice, this does not seem to be the case for humans or other species. Thymectomy and anti-T-lymphocyte serum prevent death in newborn animals (WEISSENBACHER et al. 1969a; TARATUTO et al. 1973), and nu/nu mice survive with a persistent infection (WEISSENBACHER et al. 1983a, b). Similar immunopathological mechanisms appear improbable in humans or guinea pigs; both have courses that include lymphopenia and immune depression. Direct tissue damage has been advanced (CARBALLAL et al. 1981a), but supportive evidence of an immunological cause is lacking. Several alternative mechanisms remain to be explored. For instance, it has been shown that in human AHF very high levels of circulating interferon are present (LEVIS et al. 1984). Since in LCM infection interferon may be responsible for tissue damage (GRESSER 1982), this possibility should be considered. PMN leukocytes may also play a role in the pathogenesis of lung lesions and in disorders of the complement and clotting systems. As already mentioned, neutropenia induced by administration of specific antiserum prevents the development of interstitial pneumonia in guinea pigs infected with lethal JV strains. Alterations in the clotting system in patients with AHF consist of an increase in the partial clotting time of kaolin-activated thromboplastin and a decrease in factors VIII, IX, XI, and XII. Kallikrein and its inhibitors are also diminished (KORDICH et al. 1982; MOLINAS et al. 1982). Some of these disorders could be attributed to release of plasminogen and other proteases from damaged PMN cells. A moderate and transient plasminogen activation has been demonstrated in sera from patients with AHF and in the plasma of guinea pigs and marmosets infected with JV (MOLINAS et al. 1978, 1983). The hemorrhagic syndrome present in human and experimental infections could be attributed to the coagulation alterations and to the marked thrombocytopenia. No evidence of intravascular coagulation (GONZÁLEZ et al. 1980), nor immune complex disease was found (COSSIO et al. 1975). Activation of the complement system could also participate in the pathogenesis of the clotting abnormalities and in damage to the microvasculature. Total serum C2, C3, and C5 were reduced, although C4 was increased. C3 degradation production was found in patients with AHF, for which activation of the alternative complement pathway was postulated (BRACCO et al. 1978). In guinea pigs infected with the XJ strain, activation of complement through the classical pathway was observed (RIMOLDI et al. 1977). Since no immune complexes were present, it was suggested that complement activation could be induced by substances similar to lysosomal leukocyte extracts (RIMOLDI and

DE BRACCO 1980). In humans and guinea pigs alterations of the complement and clotting systems appear simultaneously, suggesting the presence of a shared activating factor. This is not the case in marmosets infected with the XJ strain; complement activation appears early in the course of the disease, and complement returns to normal levels before clotting alterations appear (MOLINAS et al. 1981, 1983).

At the present time the neurological syndrome, present to a variable degree in most AHF patients, has no adequate pathogenic interpretation. Characteristic morphological alterations and infective virus have not been found in patients dying with severe neurological manifestations, in contrast to the experimental infection of marmosets, with their marked meningoencephalitis and active virus replication in the brain. The finding of autologous immune globulins and JV antigens in the brain's microvasculature in humans suggests some kind of immunecomplex damage (COSSIO et al. 1977), but because of the few cases examined more intensive studies are warranted.

6 Treatment and Prevention of AHF

6.1 Serotherapy

A few years after the description of AHF and the identification of JV, attempts to treat patients with plasma or serum containing anti-JV neutralizing antibodies were undertaken. In humans treatment with plasma from patients recovered from AHF apparently decreased mortality. These pioneer studies were based on the observation that immune serum used to treat guinea pigs infected with a lethal strain of JV improved survival (WEISSENBACHER et al. 1968). However, plasma antibody was not assessed, and etiologic diagnosis by virus isolation or seroconversion was seldom performed on treated patients or plasma donors. In 1979 the first controlled study on the effect of convalescent plasma on AHF progress was reported (MAIZTEGUI et al. 1979). When treatment was started before day 8 after onset of symptoms, mortality was reduced from 16% to 1%–2%. Efficacy was significantly decreased when immune plasma was administered later. In treated patients viremia dropped within 24 h, and clinical symptoms and hematologic alterations were less severe than in control cases receiving nonimmune plasma. Efficacy of treatment also depended on the amount of antibody administered. This finding led to dose standardization (ENRÍA et al. 1984). Although treatment significantly improved AHF prognosis, a new syndrome appeared in approximately 10% of treated cases. At about 4–6 weeks the disappearance of symptoms, some patients presented with neurological manifestations such as dizziness, diplopia, cerebellar ataxia, disarthria, headache, vomiting, and nystagmus associated with moderate fever. In most cases the disease was self-limiting and patients recovered in 1–3 months. Death was the exception; the single fatality reported had ascending paralysis and respiratory failure. Most patients recovered completely, although dizziness, nystagmus, and hyperexcitability could persist for up to 4 months (MAIZTEGUI et al. 1979; ENRÍA

et al. 1986). Recently, by means of evoked potential responses, mild permanent damage to acoustic centers was found in a small group of patients, suggesting that in spite of clinical recovery the CNS remains affected to some extent (CRISTIANO et al. 1985). The pathogenesis of this late neurological syndrome is unknown, although several hypotheses have been put forward. On the basis of clinical and experimental evidence, the possibility of a persistent infection was considered despite the fact that patients have antibodies in their sera and no virus can be recovered from peripheral blood. In support, when guinea pigs are infected with the XJ strain and treated with anti-JV serum, the time of death is delayed several days and animals die with meningoencephalitis and high titers of virus in the brain (LAGUENS et al. 1984). In untreated animals virus is absent in the brain and death results from bone marrow necrosis and interestitial pneumonia. In rats treated with immune serum an increased survival associated with delayed synthesis of anti-JV antibodies and presence of JV in brain was reported (AVILA et al. 1982). The appearance of a persistent infection in the CNS of marmosets treated with immune serum has already been mentioned. Although all these experimental findings point out that treatment with immune serum modifies the course of infection, either by facilitating the access of virus to the brain or by modifying the immune response, permitting a more prolonged infection, these results cannot be extrapolated to the human disease. An immunopathologic mechanism has also been considered, since patients with late neurological syndrome have anti-JV antibodies in the cerebrospinal fluid and serum antibody titers are significantly higher than in plasma-treated patients with out the late syndrome (ENRÍA et al. 1986). Alternatively, the infective virus in these cases could be intrinsically neurotropic or else these patients would have died in any case without convalescent plasma treatment. Due to the low mortality, no postmortem studies have been available up to now to provide further data for interpreting this novel iatrogenic disease.

6.2 Chemotherapy

As mentioned above, passive administration of plasma from convalescent patients is the only specific therapy available for AHF. Some chemotherapeutic drugs such as bis-benzimidazol, or amantadine proved effective only in vitro against JV, but their action has so far been discouraging in experimental animals (COTO et al. 1969; LEON et al. 1976, 1977).

Ribavirin is a nucleoside analogue with a broad antiviral spectrum and low toxicity when administered orally or parenterally to mammals (SIDWELL 1980). A large number of DNA and RNA viruses are sensitive to this compound both in vitro and in vivo. Pichinde, Machupo, and Lassa arenaviruses are also sensitive to ribavirin, as shown by experiments in rodents and primates (HUGGINS et al. 1984), as well as in clinical trials of patients suffering from Lassa fever (MCCORMICK et al. 1984).

The use of ribavirin against JV has also been reported in vitro where the virus proved sensitive, but in the guinea pig model there was merely a delay in the day of death with late paralysis and no survival (HUGGINS et al. 1984).

In order to quantify the effect of ribavirin on JV infection in vitro, different concentrations of the drug were evaluated in infected Vero cells. JV replication was affected by low concentrations of ribavirin, as shown by the fact that 3 μg/ml of the drug fully inhibited the CPE in Vero cells; both virus yield and antigen formation were reduced to undetectable levels when the concentration of the drug was 25 μg/ml (M. RODRIGUEZ et al., unpublished).

To assess the effect of the antiviral ribavirin on the course on JV infection of the marmoset *C. jacchus,* seven inoculated monkeys were treated with 15 mg/kg twice a day, starting 6 days after infection when well all animals were viremic.

The three untreated controls showed typical signs of JV virus infection 14 days postinfection and died, with the mean time of death at 18 days. In contrast, no signs of illness were detected in ribavirin-treated infected animals up to 24 days postinfection, when six of the seven marmosets showed signs of neurological involvement, such as gait lateralization, lassitude, and front or hind limb paresis or paralysis. In fact, five of these animals died with the mean time of death at 36 days, while the two remaining treated monkeys improved and survived infection without sequelae, for a total survival rate of 28% (WEISSENBACHER et al. 1985b).

Viremia with titers ranging from 1.7 to 2.8 $\log_{10}$ LD50/ml was present in all animals at the time the treatment with ribavirin began (6 days postinfection). Higher values were detected during the 3rd week postinfection, but no significant difference was observed between blood viral titers in treated and untreated marmosets during this period. During the 4th week postinfection, lower blood viral titers were present in animals surviving infection as compared with nonsurviving, treated marmosets. No virus was rescued from blood samples taken up to 100 days postinfection from the survivors.

Levels of serum-neutralizing antibodies were somehow predictive of the outcome of infection. Treated monkeys with no detectable antibodies at 25 days postinfection were the first to die; animals showing limited humoral responses at that time died around 40 days postinfection; the two marmosets presenting higher levels of neutralizing antibodies at 25 days postinfection survived.

An attempt to increase survival by administering a high ribavirin dose (50 mg/kg/day) by the same schedule proved unsuccessful, since the survival rate barely reached 20%. This lower protection could be attributed to drug toxicity at this higher dose, as hematological studies in drug control monkeys showed severe anemia and leukopenia.

Up to now, ribavirin has been the only drug shown to increase survival or cause a significant delay of death in JV-infected primates. In this model, therapy prevents clinical illness and death during the acute phase by protecting the host from systemic invasion, but appears to favor delayed viral neurotropism. The absence of more significant protection and the later appearance of neurologic involvement in almost all treated animals may also be ascribed to inadequate drug concentration in the CNS, or to brain viral replication in this animal model in contrast to humans. For this reason it is quite likely that properly scheduled ribavirin treatment for clinical AHF may prove more successful, since human CNS seems not to be the target organ for JV replication, at least in the acute phase, as shown by the rarity of rescuing JV from patients,

even those presenting overt neurologic involvement (MAIZTEGUI et al. 1982; M.R. LAGUENS, unpublished). At any rate, the potential neurotropism of JV should be borne in mind as a permanent risk when testing new therapeutic procedures.

6.3 Vaccines

Several attempts have been made to develop an effective antigen for immunization against JV, following four main lines of research: the use of inactivated virus, a subviral antigen, attenuated homologous virus, and nonpathogenic heterologous virus.

Inactivated antigens have been prepared by UV irradiation (D'AIUTOLO et al. 1979), by employing neutral red (PARODI et al. 1965) or methylene blue followed by photoinactivation (MARTÍNEZ SEGOVIA and DE MITRI 1977), or else by means of formaldehyde, acetone, or heat treatment (BARRERA ORO et al. 1967; CARBALLAL et al. 1985). However, guinea pig inoculation with these antigens and later challenge with pathogenic strain has not yet proved satisfactory.

As stated in Sect. 2.2, the glycoprotein envelope G38 is able to induce serum-neutralizing antibodies and protection against lethal infection in the guinea pig (CRESTA et al. 1980).

As regards attenuated virus, a highly passaged XJ prototype strain was cloned in MA-111 cells, yielding XJCl3, which was attenuated for guinea pigs and conferred long-lasting immunity with high titers of neutralizing antibodies (GUERRERO et al. 1969). This step led to the development of a seed virus vaccine in suckling mouse brain and to the first trials in volunteers. As a start, seven professional staff members involved in AHF research received the XJCl3 strain (WEISSENBACHER et al. 1969b). Gradually, over a 2-year period, 636 healthy volunteers from the endemic area were likewise immunized, most of whom developed a subclinical infection and one-third a mild transient fever on occasion with myalgia, asthenia, or headache (RUGGIERO et al. 1974). Neutralizing serum antibodies persisted in 90% of volunteers up to 9 years postvaccination (RUGGIERO et al. 1981).

Owing to the fact that this vaccine was prepared in suckling mouse brain and the virus had been cloned in heteroploid cells, its administration to volunteers was discontinued. Attempts were then made to replicate and clone the virus on other substrates, such as diploid cells suitable for human use. Thereby, a deeper knowledge of attenuated strains, their advantages and drawbacks was gained.

An alternative attenuated strain (XJO) derived from the same parental line (XJ prototype strain) as XJCl3, behaves likewise in experimental animals, but offers the advantage of not having been passaged in heteroploid cells (GUERRERO and BOXACA 1980; GUERRERO et al. 1983). It has been reported to confer complete protection against pathogenic challenge in guinea pigs. However, infectious virus in organs could be detected by coculture with Vero cells, up to 60 days postinfection for XJO or 90 days postinfection for XJCl3 in the lymphohemopoietic organs and CNS, thus indicating persistent infection with either of these attenuated strains. (MALUMBRES et al. 1984; GUERRERO et al. 1983). As pre-

viously shown for Tacaribe virus, the XJO attenuated strain is able to confer protection as early as 3 days postinfection, and this early protection is also attributed mainly to viral interference (GUERRERO et al. 1985).

Another attenuated strain (Candid 1), derived from the same parental XJ strain, has been developed as a potential vaccine by cloning and replication in FRHL-2 cells. Standard tests for neurovirulence have demonstrated no significant adverse effects in rhesus monkeys and guinea pigs (LUPTON et al. 1984); additionally, serological and challenge studies have shown its efficacy in the same animals models (KELLY et al. 1984). No virus or antigen was detected in organs from *Rhesus macaques* at 7 months after intramuscular inoculation of Candid 1, and monkeys exhibited no virus-induced CNS lesions at this time. Development of neutralizing antibodies correlated with the disappearance of viremia and was detected earlier in animals receiving higher virus doses (BARRERA ORO et al. 1985).

XJC13 infection of the marmoset *C. jacchus* resembles human vaccination as regards clinical features and the long-lasting presence ot serum antibody (AVILA et al. 1985). Viremia was present from days 6 to 22, titers peaking at 4.0 logs. Viral spread was limited to the lungs, spleen, lymph nodes, and bone marrow on day 14. No virus was found in organs on day 23, and neither hematologic alterations nor pathologic lesions were seen in these monkeys. Antigen was detected by IF in lymph nodes, spleen, adrenals, lung, and brain.

Neutralizing antibodies were detected from the 3rd week onward. Protection conferred by the XJC13 strain proved effective when animals were challenged with the pathogenic XJ strain at days 60 or 380 postinfection, whereas 100% of controls died. When viral persistence was sought by coculture on days 370, 390, and 420 postinfection, no infectious virus was detected.

As a rule, XJC13 strain replicates in guinea pigs less efficiently than XJ strain and causes 16% mortality (AVILA et al. 1979), but there is a significant increase in both viral replication and mortality when animals are immunosuppressed with cyclophosphamide (SAMOILOVICH et al. 1982). Guinea pigs infected with the attenuated strain XJ44 and treated with cyclosporin A or cyclophosphamide succumbed to AHF with high viral titers and histopathological lesions similar to those induced by the virulent strain (KENYON et al. 1985). Taken together, these findings serve as a warning against vaccination of immune-suppressed patients with JV-attenuated strains.

After intracerebral inoculation of guinea pigs (BOXACA et al. 1982) or the primate *Cebus* sp. (CARBALLAL et al. 1982) with XJC13 strain, a certain degree of neurovirulence became manifest. However, cebus monkeys surviving infection with pathogenic or attenuated JV strain by intramuscular or intracerebral routes exhibited no viral persistence in organs, even following treatment with immunosuppressors (OUBIÑA et al. 1984b).

The neurotropism of attenuated and pathogenic strains is affected by the passage history of the virus in guinea pigs. In this system, attenuated strains apparently have a higher neurotropic potential than the pathogenic prototype of JV (BOXACA et al. 1984a).

It was demonstrated that in pregnant guinea pigs, the attenuated XJC13 strain reached the fetus by the transplacental route either during acute or chronic infection (BOXACA et al. 1984b; GOMEZ and BOXACA 1985).

The risk of neurotropism, viral persistence, and transplacentary infection, together with the demonstrated higher risk for the immunosuppressed host, explain why the use of heterologous cross-protection with nonpathogenic viruses was considered in the 1960s (PARODI et al. 1964; TAURASO and SHELOKOV 1965) and could still offer an alternative approach to AHF prophylaxis.

Cross-protection studies between JV and five other Tacaribe complex viruses showed that previous infection with Tamiami or Pichinde delayed guinea pigs deaths, and 58% survival was found in animals immunized with Amaparí. Furthermore, one dose of Machupo or Tacaribe virus fully protected against challenge with JV (WEISSENBACHER et al. 1976b). In an attempt to resort to cross-protection against JV, Tacaribe virus has been widely assayed in guinea pigs (DAMONTE et al. 1978b; COTO et al. 1980) and monkeys (WEISSENBACHER et al. 1982; SAMOILOVICH et al. 1984) as a potential vaccine.

Tacaribe virus is the most closely related to JV, seems not to be pathogenic for humans, marmosets, or guinea pigs, and induces long-lasting cross-protection against JV in animal models (WEISSENBACHER and DAMONTE 1983). In guinea pigs infected with Tacaribe virus, neither viral persistence nor chronic lesions developed, and long-lasting cross-protection against JV was achieved, i.e., 100% of the animals were protected up to 18 months after a single inoculation (G. CARBALLAL et al., unpublished).

Following intramuscular inoculation with Tacaribe virus, the marmoset *C. jacchus*, for which JV is fatal, exhibits no clinical signs, no viremia, and no alterations in hemoglobin, hematocrit, reticulocyte, erythrocyte, platelet, or leukocyte values (WEISSENBACHER et al. 1982). This animal thus gains 100% protection against lethal JV doses, lasting up to 8 months, and no virus is detectable in the organs, even by coculture with permissive Vero cells. Protection had dropped at 16 months after Tacaribe inoculation, since one out of three monkeys challenged at that time died and pathogenic virus was rescued from their organs (M.C. WEISSENBACHER, unpublished).

The mucosal route also proved effective for Tacaribe immunization against pathogenic JV in marmosets. All primates inoculated by the nasal route developed neutralizing serum antibodies without clinical signs and were protected against lethal challenge. Neither neuropathogenicity nor virus persistence could be detected even after intrathalamic inoculation of Tacaribe virus in *C. jacchus* (SAMOILOVICH et al. 1984). These results closely correlated with the lack of pathogenicity of Tacaribe virus previously observed in intracerebrally inoculated guinea pigs.

Many studies have been carried out to determine the degree of pathogenicity of JV strains. However, there are no effective in vitro markers to differentiate attenuated from pathogenic strains, and up to now the in vivo markers have been the most widely employed, such as the guinea pig, mouse, and primate models (Fig. 1).

Initially, the laboratory animal most frequently used to differentiate attenuated and virulent strains of JV has been the adult guinea pig (GUERRERO et al. 1969). The virulence of JV strains for humans and guinea pigs seems to correlate closely, and therefore the guinea pig has been widely used as a model to study virulence patterns.

The differences in the efficiency of lethal infection of 11-day-old guinea pigs inoculated by the intramuscular route suggest that this system may also be appropriate as a marker for JV virulence (CONTIGIANI and SABATTINI 1984). The 14-day-old mouse inoculated intracerebrally is currently used to differentiate the attenuated from the pathogenic strain (CONTIGIANI and SABATTINI 1977). An alternative attenuation marker is a 2-day-old rat. When infected by the intracerebral route with the pathogenic XJ strain, mortality is nil (NEJAMKIS et al. 1977). whereas XJCl3 proves lethal (AVILA et al. 1981a). Remarkably, intraperitoneal inoculation leads to an opposite outcome exhibiting the same susceptibility as guinea pig: high mortality occurs with XJ while there are few deaths with XJCl3 (AVILA et al. 1981a). The greatest differences in mortality rates were achieved with 1000 $TCID_{50}$ of either strain by either route.

Clinical, virological, and immunological parameters of the infection in two species of primates have also been employed as virulence markers. It was shown that the marmoset *C. jacchus* may be used as a biological attenuation marker for both JV strains and Tacaribe virus, given its contrasting behavior as regards mortality, viremia, organ viral dissemination, hematologic alterations, and antibody response.

Primates infected with the XJ pathogenic strain showed hemorrhagic and neurologic manifestations including petechiae, ecchymoses, erythematous rash, tremors, and convulsions, and virus was readily recovered from blood and organs in high titers, while hematologic alterations appeared as from day 7 postinfection. Mortality was 100% with severe leukopenia, thrombocytopenia, absence of antibodies, and widespread tissue lesions at necropsy (FRIGERIO et al. 1982). On the other hand, no deaths, clinical signs, or hematologic alterations were observed in animals inoculated with the attenuated XJCl3 strain. Viremia was transient and lower than the pathogenic strain, viral spread was limited to a few organs, and the marmosets promptly developed humoral antibodies. When monkeys received Tacaribe virus, there were no clinical signs, hematologic alterations, or mortality whatsoever. Both viremia and viral spread were lacking, but serum antibodies appeared after the 2nd week.

Accordingly, this primate was proposed as an experimental model to distinguish attenuated from pathogenic strains of JV and other arenavirus (WEISSENBACHER et al. 1985c). Besides, *C. jacchus* is a neotropical primate, relatively easy to obtain and maintain in captivity at reasonable cost.

Another primate model for AHF, *Rhesus macaques*, closely mimics the human clinical picture and may serve as a marker. With the attenuated Candid 1 strain there where no overt signs of disease and the mean body weight decreased only slightly following infection. Between 1 and 21 days postinfection virus was detected by coculture of peripheral blood mononuclear cells and from serum only on day 4 postinfection. Hematologic parameters did not vary from control values at any time during the observation period. Peak neutralizing antibody titer was reached in all primates within 2 months. In contrast, rhesus infected with the virulent JV strain developed anorexia, lassitude, diarrhea, rash, and mucosal hemorrhage, as well as leukopenia, thrombocytopenia, and 30% body weight loss. Viremia levels peaked at 10^4 PFU, virus was shed from the oropharynx, and primates suffered 75%–100% mortality (BARRERA ORO et al. 1985).

As stated above, roughly 90% of the 1.5 million inhabitants residing in the so-called endemic area are still prone to contract JV infection. The steady spread of this zone towards the northwest as shown by reports of clinical cases, together with pathogenic virus rescue in rodents from nonendemic areas towards the south, indicate that the population exposed to AHF is tending to increase rather than diminish. Therefore, there is a need to develop a safe and effective vaccine for the population at risk.

7 Concluding Remarks

AHF currently represents a health problem, but it may become an even greater risk in the future. The number of clinical cases shows no tendency to decrease, and the endemic area is steadily growing. Moreover, the presence of pathogenic virus in rodents living in nonendemic areas and the knowledge that cricetids other than the known reservoirs can be persistently infected with JV increases the likelihood of more severe spreading.

AHF may be preventable by immunization of the population at risk, together with rodent control, either by decreasing the number of reservoirs or altering the pattern of their persistent infection. Progress has been made in developing live vaccines during the past few years, but further data on reservoir ecology and behavior are essential to elucidate the mechanisms of JV persistence in nature.

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Neutralization of Arenaviruses by Antibody

C.R. HOWARD

1 Introduction

Neutralization of infectivity is the end result of a number of alternative or synergistic reactions between a virus and antibody directed against certain antigenic determinants on its surface. These reactions prevent either the attachment of virus to a particular host cell by steric hindrance, the entry of virus into the cell, or the uncoating of the virus particle to release the viral genome. In turn, the relative contribution of each of these to neutralization is dependent both on qualities of the viral antibodies (their relative affinities and immunoglobulin class) and specificity for relevant antigenic determinants (so-called critical sites). These considerations have been the subjects of several extensive reviews (MANDEL 1979; DELLA-PORTA and WESTAWAY 1978).

Extensive serological cross-reactivity can be demonstrated by complement-fixation methods among many members of the arenaviruses, although the degree of this relatedness has proved difficult to assess in either convalescent or hyperimmune sera. All available evidence indicates that the complement-fixing antigen is associated with the internal nucleocapsid, polypeptide NP (BUCHMEIER et al. 1977). In contrast, where applicable, the detection of neutralizing antibody directed against outer glycoprotein structures has proven much more specific. For example, Junín and Machupo viruses from the New World, the etiological agents of Argentine and Bolivian hemorrhagic fevers, are quite distinct in neutralization tests although closely related by complement fixation (JOHNSON et al. 1973). A similar marked specificity of neutralization has been demonstrated with antisera to lymphocytic choriomeningitis (LCM) and Lassa viruses, both Old World arenaviruses. Each may be readily distinguishable from the other

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by this technique, although conventional plaque reduction tests in vitro are more difficult to perform with either.

The role of neutralizing antibody in vivo is unclear in both human and animal arenaviruses infections. However, immune plasma characterized as containing a certain level of neutralizing antibodies has been used successfully in the treatment of patients with Argentine hemorrhagic fever, and a similar approach has been advocated for the treatment of Lassa fever. This review outlines these findings in the context of neutralizing antibody as a marker of immune plasma efficacy and the use of neutralizing antibody reactions for the immunochemical characterization of arenavirus proteins.

2 Detection of Neutralizing Antibodies and the Serodiagnosis of Human Arenavirus Infections

Early attempts to detect arenavirus-specific neutralizing antibodies consisted of injecting LCMV and human serum mixtures into the footpads of susceptible mice. The presence of neutralizing antibody prevented the development of a generalized infection with the result that the animals remained susceptible to a subsequent intracerebral challenge (HOTCHIN 1962). Although results could not be obtained in less than 3 weeks, the technique was found to be much more sensitive than direct intracerebral inoculation of virus-antibody mixtures. A more satisfactory method is the microneutralization procedure, whereby results may be obtained in less than 1 week (HOTCHIN and KIRSCH 1975). Optimal performance of this technique requires the mixing of a fixed concentration of virus with dilutions of antibody directly in the wells of microtiter trays, the addition of agarose, and then the further overlay of BHK-21 cells. This method is at least twice as sensitive as immunofluorescence for the detection of LCMV-specific antibody, with small plaques becoming apparent after 4 days. Although immunofluorescence offers considerable advantages for the rapid diagnosis of several human arenavirus infections, the sharp decline in antibodies detected by this technique makes an assay for neutralizing antibodies preferable for large scale seroepidemiological studies. It should be noted that alternative techniques employing constant antibody and varying virus concentrations for the determination of log neutralization indices are also subject to several drawbacks (FAZEKAS DE ST GROTH 1962), and quantitation is best accomplished by using mixtures containing a constant virus concentration and varying dilutions of samples containing antibody. For example, LEHMANN-GRUBE (1971) described the quantitation of neutralizing antibodies by determining the reciprocal of that serum dilution which reduces virus infectivity by 2 logs.

The detection of neutralizing antibodies may also be of value in the diagnosis of Bolivian and Argentine hemorrhagic fevers. WEBB et al. (1969) described neutralizing antibodies as appearing during the acute phase of Machupo virus infection and persisting for at least 12 months. In contrast, complement-fixing antibodies in patients with either disease are comparatively short-lived and indicative of only recent or ongoing infections.

The sensitivity and specificity of neutralization tests for detecting JV antibodies have been exploited in a number of studies for the retrospective diagnosis of subclinical infections (for example, TEYSSIE et al. 1971; WEISSENBACHER et al. 1980). The test may be carried out by using the constant serum and varying virus concentration method, revealing inapparent infections in approximately 20% of laboratory workers handling known or presumed positive specimens.

The specificity of neutralization methods have also been used to dissect antibody responses to JV and LCMV, since these arenaviruses may coexist in the rodent population within the endemic region of Argentine hemorrhagic fever (MAIZTEGUI et al. 1972; SABATTINI et al. 1974). A study of nearly 3000 cases of Argentina hemorrhagic fever showed that approximately 0.2% of these patients were, in fact, infected with LCMV and that over 5% of all acutely ill patients have some serological evidence of exposure to LCMV (BARRERA ORO et al. 1977). In the latter group, JV may be successfully isolated during the acute phase of hemorrhagic fever despite the concomitant presence of neutralizing antibodies to LCMV.

A recent study has shown that antibody titers may in part be related to the host-cell type used for the growth of virus, with higher neutralizing antibody titers being obtained if tests are performed using sera prepared with virus grown in cells of the same species as their used for immunization (COULOMBIE et al. 1984). These authors have suggested that recruitment of host antigens into the viral envelope at maturation may enhance the immune response in the homologous host, at least in the case of responses in guinea pigs to JV or Tacaribe virus.

It is well recognized that high titered antisera against either Lassa virus or the nonpathogenic Pichinde virus posses minimal neutralizing capacity when examined by the mouse neutralization test (LEHMANN-GRUBE et al. 1979) or tissue culture tests (BUCKLEY and CASALS 1970; SENGUPTA and RAWLS 1979). 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 from Lassa virus-infected patients is unclear, but the studies of CHANAS et al. (1980) showed clearly that neither aggregation nor extensive sensitization of Pichinde virus particles occurs after reaction with homologous antiserum. The presence of antibodies directed against the major structural protein of the virus did not prevent adsorption and penetration of virus antibody complexes into the host cell.

3 Cross-Reactions with Neutralizing Antibodies

There is some evidence that JV is weakly neutralized by animal hyperimmune serum against Tacaribe virus (HENDERSON and DOWNS 1965; WEISSENBACHER

et al. 1975/1976), and further evidence that immunization of guinea pigs with Tacaribe virus protected against challenge with virulent JV (COTO et al. 1976; TAURASO and SHELOKOV 1965).

Various degrees of protection were also observed using other nonpathogenic members of the Tacaribe complex, the most effective being Amapari virus, which induced protection in nearly 60% of guinea pigs and extended the survial titers of the remainder after challenge with JV. The protective effect of Tacaribe virus may be demonstrated as early as 3 days after immunization. Detailed analysis of the neutralizing antibody response in animals protected by heterologous vaccination has shown that neutralizing antibodies to Tacaribe virus appear by day 10 and that subsequent challenge with JV stimulates the production of antibody specific for JV with all the characteristics of a secondary antibody response (COTO et al. 1980). Repeated stimulation of the guinea pig immune system with Tacaribe virus induced a high antibody titer against the homologous virus; during this time heterologous reactions against JV developed steadily, although the highest responses never exceeded or approached that specific for Tacaribe (WEISSENBACHER et al. 1975/1976).

The protective effect of vaccination with Tacaribe virus appears to result from priming of the immune system by Tacaribe viral antigens bearing a close resemblance to the relevant determinants of JV glycoproteins. Animals inoculated with Tacaribe virus alone develop cross-reactive neutralizing antibodies to JV 8 weeks later, but challenge with JV stimulates the appearance of a typical secondary antibody to it (DAMONTE et al. 1978). This anamnestic response is seen on challenge also with inactivated JV and confirms both that this response is essentially antigenic in nature and that JV replication is not required. A similar heterologous neutralizing response has also been documented in Tacaribe-inoculated marmoset monkeys (*Callithrix jacchus*) challenged with JV (WEISSENBACHER et al. 1982). After challenge there was a brisk response to JV, although the titers of neutralizing antibody against Tacaribe virus remained relatively constant.

4 Experimental Studies with Monoclonal Antibodies

BUCHMEIER (1984) has reported neutralization of LCMV 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 a limited sharing of epitopes involved in the relevant antigenic site between closely related viruses. These antibodies all react with the large GP1 envelope glycoprotein of the virion, a result in agreement with the observations of BRUNS et al. (1983) 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

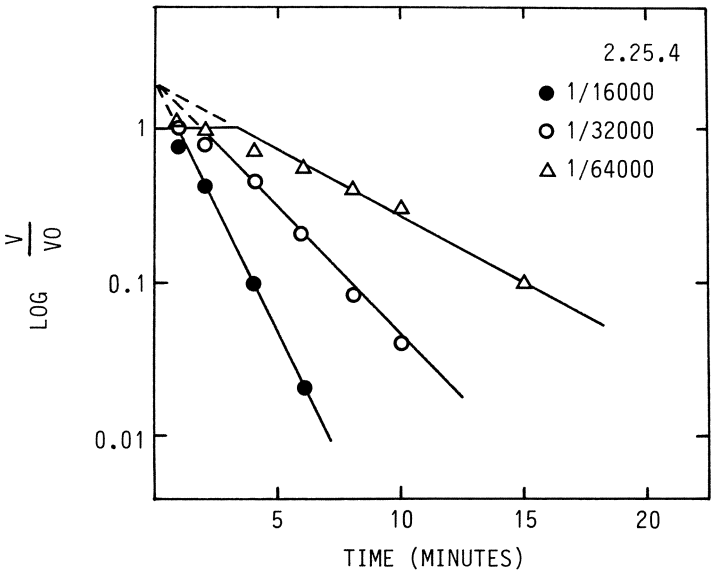

Fig. 1. Kinetic neutralization of Tacaribe virus with antibody (2.25.4) at 30° C. Dilutions of antibody examined were 1:16000 (●), 1:32000 (○), and 1:64000 (△). Reduction in infectivity was plotted as the $\log_{10}$ value of residual virus (*V*) divided by titer at time 0 (*VO*). Extrapolation of plots at each dilution indicates multihit kinetics (from HOWARD et al. 1985)

at high dilution, although no reactions were recorded against the closely related JV. 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-JV sera failed to neutralize Tacaribe virus whilst anti-Tacaribe immune ascites neutralized both JV 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 incubation. At high dilution, one antibody (Fig. 1) neutralized all infectivity after an initial lag period, suggesting that two or more molecules of antibody are required for neutralization, since the single-hit theory predicts a linear respone from the time of mixing virus with antibody (DELLA-PORTA and WESTAWAY 1978). The remaining antibodies gave significant nonneutralized fractions after an initial linear response (Fig. 2). Residual virus infectivity was decreased by the 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 caused by steric hindrance from nonneutralizing 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

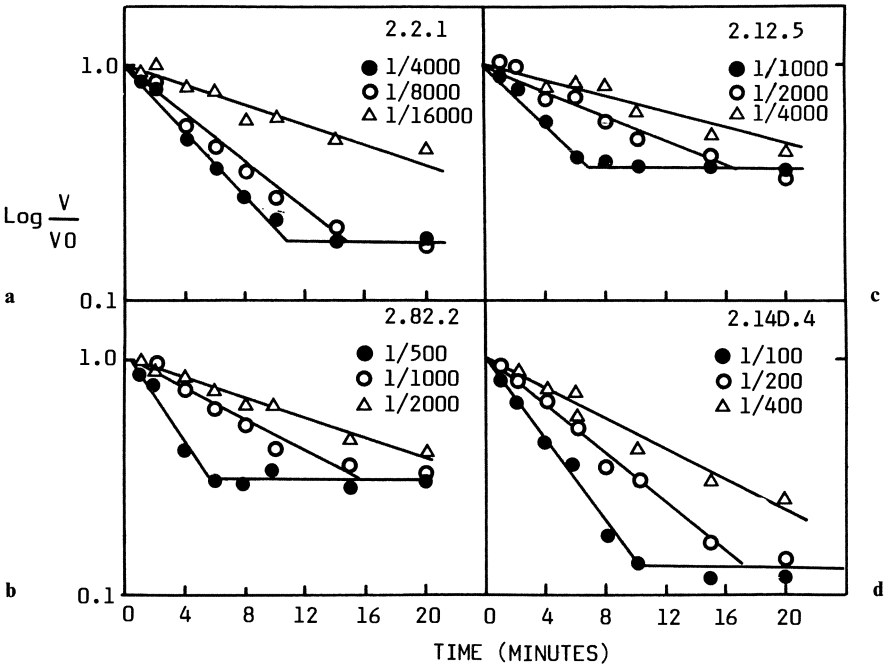

Fig. 2. Kinetic neutralization of Tacaribe virus with antibodies directed against the surface glycoprotein, but producing a large resistant fraction. Each panel represents reactions with a separate antibody each of which recognizes similar or overlapping epitopes. Antibodies were used at twofold dilutions (● to ○ to △) starting from an initial dilution of (**a**) 1:4000, (**b**) 1:1000, (**c**) 1:500, and (**d**) 1:100 (from HOWARD et al. 1985)

Table 1. Heterologous neutralization reactions between variants of Tacaribe virus resistant to monoclonal antibody 2.25.4 and neutralizing antibodies that recognize a second unrelated antigenic determinant

	Antibody				
	2.25.4	2.14.D4	2.2.1	2.12.5	2.82.2
Parent	+[a]	+	+	+	+
V2/1	−	−	−	(+)[b]	+
V2/2	−	+	−	−	+
V2/3	−	−	−	(+)	+
V2/10	−	+	+	+	+
V2/11	−	−	+	+	+
V2/12	−	−	+	+	+
V1/1	−	+	+	+	+
V1/3	−	−	+	+	+
V1/5	−	−	+	+	+
V1/6	−	+	+	+	+
V1/7	−	−	+	+	+

[a] Infectivity reduced by one $\log_{10}$ or more after 15 min at 37° C
[b] Infectivity reduced by one half $\log_{10}$ after 15 min at 37° C

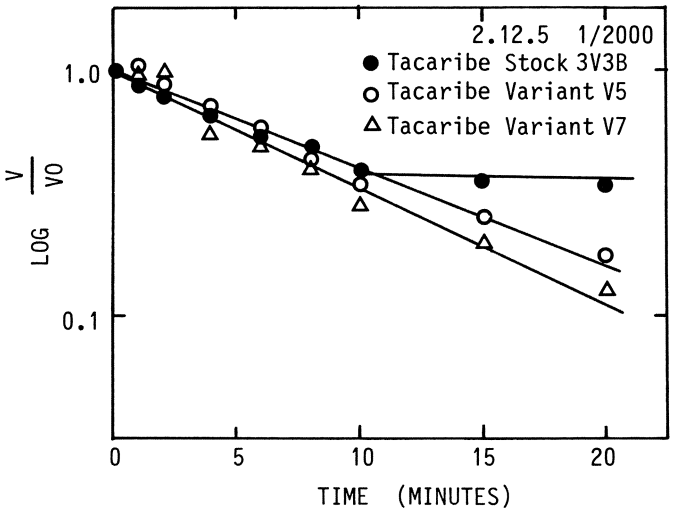

Fig. 3. Reaction of Tacaribe virus variants selected for resistance to antibody 2.25.4 (see Table 1). Variants V1.5 (o) and V1.7 (△) showed enhanced neutralization with antibody 2.12.5 when compared with the parental virus (●) (from HOWARD et al. 1985)

neutralization of Newcastle disease virus with monoclonal antibodies suggests that lower avidity does not necessarily result in a larger nonneutralized fraction (IORIO and BRATT 1984). In the latter study, however, a mixture of monoclonal antibodies drected against four distinct epitopes on the virus surface showed neutralization kinetics similar to those obtained with animal polyclonal 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}. The variants may be clearly differentiated into two or more groups by the complete absence of neutralization in the presence of neutralizing monoclonal antibodies recognizing distinct epitopes on the Tacaribe virus glycoprotein (Table 1). Some variants were neutralized by one monoclonal antibody (2.12.5) to a much greater degree than by the parental virus, with a linear decrease being observed over the whole 20-min period of observation (Fig. 3). Reaction of the variants with the heterologous monoclonal antibodies manifesting incomplete neutralization suggests that point changes occuring in or around the epitope recognized by the selecting antibody may alter the configuration of nonoverlapping 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 (Table 1).

5 Neutralization In Vivo: The Use of Immunoglobulin and Passive Immunization

The use of nonhuman primates in studying the pathogenesis of hemorrhagic fevers has included the evaluation of passive prophylaxis in experiments with immune plasma of human origin. EDDY et al. (1975) have described an immunoglobulin preparation obtained after Cohn fractionation of pooled sera from 14 patients with Bolivian hemorrhagic fever. All had histories of Machupo virus infection and contained neutralizing antibodies against the virus with a dilution end-point of 1:128. After purification, the final immunoglobulin preparation had a titer of 1:2048 and a protein concentration of 167 mg per 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. Protection was significant in animals receiving 0.5 ml or more immunoglobulin per kg 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 detectable by 21 days in monkeys 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 encephalitis with an abrupt onset, without hemorrhagic signs, but invariably fatal within 4–6 days. No viral antigen could be detected by immunofluorescence in the tissues of animals with the neurological syndrome. 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 it is unclear whether antigen-antibody complexes played any role in the late development of encephalitis or some unrelated viral factor was responsible. Whether this syndrome is similar to the spontaneous encephalitis occasionally seen in acutely infected primates is also unknown. 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 while studying animals infected with specimens from a donor with 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 immune plasma was administered. The source was a missionary nurse who had been 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 complement-fixing titer in excess of 1:32 and capacity to neutralize at least 100 $TCID^{50}$ of infectious virus. Despite the patient's high titer of specific antibody, the masking of residual infectious virus was a prime consideration, particularly since the diagnosis of Lassa fever had not been confirmed

at that time. However, the patient began to improve rapidly within 24 h of receiving the plasma, becoming afebrile 7 days later. Although laboratory diagnosis of Lassa fever 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 receipt of the convalescent plasma, although virus continued to be isolated from throat washings for at least 2 weeks and from urine for more than 1 month after beginning treatment. This illustrates that particular care must be taken in monitoring virus in body fluids for some time after a patient enters convalescence, even if immune plasma has been used therapeutically. In this instance, complement-fixing antibody was not detected in significant amounts until the 52nd day after the onset of illness.

MONATH et al. (1974) reported the use of immune plasma in two acutely infected patients with Lassa virus admitted to the Panguma Catholic Hospital, Panguma, during the 1972 outbreak in Sierra Leone. Both patients received 250 ml 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. The first of these patients, a 20-year-old woman, was admitted approximately 14 days after the onset of symptoms. At the time of plasma administration, her oral temperature was 101.2° F; its decline to 98.6° F after 12 h 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. This 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 leukocyte 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 are complicated because the disease is frequently self-limiting with rapid clinical improvement in the second or third week of illness. However, previous work had shown that the mortality in guinea pigs infected with JV may be considerably reduced by passive transfer of antibodies (CARBALLAL and FRIGERIO 1973). 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 presumed Argentine hemorrhagic fever. All these patients entered the clinical trial within 8 days after the onset of illness, and in 188 the diagnosis was confirmed by laboratory testing. Immune plasma was drawn from donors convalescent from Argentine hemorrhagic fever in whom at least a fourfold rise in antibody titer had been demonstrated previously. The results obtained by MAIZTEGUI et al. clearly showed a marked improvement in the mortality rate of patients who received immune plasma compared with the control group.

Apart from the clearly beneficial effect of the immune plasma, two important points emerged from this study. First, this improvement was seen in patients treated early in the course of illness; retrospective analysis of an additional

24 patients who received immune plasma on day 9 or later showed the same mortality rate as patients receiving normal plasma. Second, a number of patients successfully treated with immune plasma were readmitted 2–3 weeks later 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 our the parallel with the studies of EDDY et al. (1975) in monkeys experimentally infected with Machupo virus after passive immunization. As discussed above, the protected monkeys did not develop hemorrhagic illness on challenge with the virus, although many animals exhibited a late neurological syndrome some weeks later. Possibly this complication is directly related to the immunoglobulin dose, at least in the case of Machupo-infected primates (EDDY and COLE 1978).

In a retrospective study of JV infected patients treated with immune plasma, ENRIA and colleagues (1984) determined that the neutralizing antibody titer in individual lots of administered plasma was a critical factor. Only 3 of 30 survivors so treated had received plasma with a low antibody titer. However, four of seven 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 viremia, 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 α-interferon in patients receiving immune plasma, presumably either by restricting further virus spread to macrophages or leukocytes 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 have been discussed by MONATH et al. (1974). 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 titer of specific antibody and the extent of possible antigenic variation in Lassa virus isolates. The occurrence of Lassa virus subtypes would raise the possibility of limited protection if the antibody donor and recipient were infected with heterologous subtypes. A further consideration is the screening of immune plasma for markers of viral hepatitis. For example, the endemic area of Lassa fever closely corresponds with a high carriage rate of hepatitis B virus; in some countries of West Africa, this rate 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 titer in each case exceeded 2 logs as measured in vitro by a plaque

reduction test. Similarly, MAIZTEGUI and colleagues found that immune plasma treatment of patients with Argentine hemorrhagic fever was most effective when the titer of neutralizing antibody was high (MAIZTEGUI et al. 1979; ENRIA et al. 1984). It is important to note that JAHRLING found a positive correlation between protection and neutralizing antibody titer with respect to Lassa virus, but this relationship was not maintained when immunofluorescence was used for antibody titration. Immune plasma obtained on or before the 45th day from a convalescent human failed to protect susceptible guinea pigs, even though the titer of this preparation exceeded 1:2560 by immunofluorescence. In contrast, plasma taken at 90 days conferred protection, although the immunofluorescence titer remained unchanged. In these animals there was no sign of viremia. Progressive dilution of immune plasma resulted in less than 100% protection and a corresponding rise in viremic levels from the reduction in the amount of specific antibody administered.

Efficacy testing in infected cynomolgus monkeys has shown a similar correlation between the titer 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 were 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 quality appeared to be an important variable; immune plasma with only marginally significant titers of antibody against the virus may still prove useful if infused in sufficiently large volume early in infection. Plasma diluted below the level known to confer protection did not have a significant effect on viremia, suggesting that low levels of passively administered antibody do 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 titer did not achieve the same level of protection as an equivalent single dose administered early after exposure to the virus.

In contrast to the experience with the treatment with immunoglobulin of Machupo-infected rhesus monkeys, there appeared to be no sign of 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 in comparison with a reference strain obtained originally from neighboring 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 PETERS (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 viremia, although an antibody response 6 weeks later suggested the development of a subclinical infection. Those treated later subsequently developed viremia, 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 reason for this synergism was unclear, passive immunization appeared to suppress viremia without significantly reducing the level of virus replication only in target tissues, whereas ribavirin concentrated in tissues identified as major sites of virus replication. As a result, significantly less virus was recovered from the spleen, liver, lung, and other organs (JAHRLING and PETERS 1984). Combined therapy for humans infected with Lassa virus is particularly attractive for two reasons. First, much of the immune plasma available is of low titer and its effect could be usefully enhanced by simultaneous antiviral treatment. Second, 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 viremia.

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 clearly contains antiviral antibodies. 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 complement-fixing 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 nonneutralizing 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.

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Nature of the Inflammatory Process in the Central Nervous System of Mice Infected with Lymphocytic Choriomeningitis Virus

J.E. ALLAN, J.E. DIXON, and P.C. DOHERTY

1 Introduction

Lymphocytic choriomeningitis (LCM) is an inflammatory disease that occurs in immunologically competent adult mice after infection of the brain with LCM virus (LCMV). Tremors develop in the legs, head, and tail, and characteristic extensor spasms of the hind legs are found during the terminal stage of disease, usually within 1 week of infection.

Histological examination of the brain 6–7 days after intracerebral injection of virus reveals inflammation of the choroid plexuses, the leptomeninges surrounding the brain, and also the ependyma lining the ventricles, whereas the brain parenchyma is not greatly involved. These sites of inflammation correspond to the distribution of virus-infected cells, as shown by staining with LCMV-specific fluorescent antibody (NATHANSON et al. 1975; SCHWENDEMANN et al. 1983). There are other clinical symptoms and pathological changes, but it is the severe inflammation of the CNS which distinguishes LCM from the disease that results from extraneural infection by viscerotropic strains of LCMV.

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The symptoms of LCM are not observed when the development of meningitis is prevented by experimental manipulations, such as treatment with immunosuppressive drugs or irradiation (reviewed by BUCHMEIER et al. 1980; LEHMANN-GRUBE 1982). Moreover, LCMV is a relatively noncytopathic virus producing persistent rather than acute infections in neontal and adult immunocompromised mice (reviewed by BUCHMEIER et al. 1980; LEHMANN-GRUBE 1982). These observations led to the conclusion that LCM is an immunopathological disease. Therefore, there is great interest in the types of cells that form the meningeal exudate and in an analysis of their contribution to the generation of disease or, alternatively, to the recovery from infection with elimination of virus. Many studies have implicated T cells as central to both the development of fatal LCM and to recovery from infection (reviewed recently by BUCHMEIER et al. 1980; LEHMANN-GRUBE 1982). However, the precise role of particular T-cell subsets and the requirement for other cell populations remain controversial.

2 A Quantitative Approach to the Analysis of Meningitis

The most relevant information on the function and type of cells involved in LCM must come from studies on the cells found in the meningeal exudate. These can be recovered by rinsing the brain tissue, but a rapid and simple method is collection of the CSF from the cisterna magna, which lies over the base of the brain stem (DOHERTY 1973). Large numbers of white blood cells are found in the CSF and are observed in brain tissue from day 5 postinfection, with the levels increasing exponentially as shown in Fig. 1. The integrity of the blood-brain barrier is destroyed since Evan's blue dye injected peripherally enters the CNS (DOHERTY and ZINKERNAGEL 1974). There is, however, still some selectivity of the cells allowed to enter, since large numbers of red blood cells are not commonly observed in the meningeal exudate.

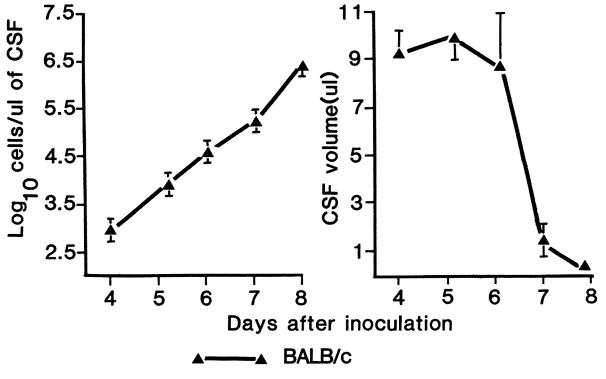

Fig. 1. Number of white blood cells in the CSF and volume of CSF obtained from groups of five BALB/c mice at various times after intracerebral infection with Armstrong (ARM) LCMV. The results are expressed as mean ± SE and are derived from data in ALLAN and DOHERTY (1985b)

At the peak of LCM, the CSF appears milky white with as many as 10^5 white blood cells/μl, but CSF from animals given an intraneural inoculation of diluent contains only background levels of cells (less than 10^2 cells/μl) within 3 days. These enormous differences in cell concentration following infection provide a very sensitive measurement of the extent of inflammation. The changes affect both the cell concentration and the volume of accessible CSF during infection. There is a dramatic decrease in CSF volume between days 6 and 7 postinfection (Fig. 1), which is accompanied by occlusion of the cisterna magna, apparently by brain stem tissue. Although suggestive of brain edema, this conclusion has not been substantiated by electron microscopy (CAMENGA et al. 1977; SCHWENDEMANN et al. 1983).

The symptoms of LCM can also be induced by intravenous transfer of immune cells to virus-infected, immunosuppressed mice. In this system there is a delay of approximately 3 days before inflammation of the CNS is observed. There is some variation between strains of mice, with CBA mice given syngeneic immune cells showing a 24-h delay in the development of severe meningitis in comparison with C57BL/6 mice.

Cyclophosphamide is commonly used to suppress the immune response of recipients prior to cell transfer. Both the dose and time of administration of this drug are critical for prevention of inflammation. Treatment with high doses (300 mg/kg) prior to virus inoculation, although effective in suppressing the immediate cytotoxic T-lymphocyte (CTL) response, appears to allow the later development of a CTL response originating from the recipient. In contrast, after doses as low as 50 mg/kg are given 5 days postinfection, CTL activity is absent on day 8 (ALLAN and DOHERTY 1985c). Administration of cyclophosphamide on days 4–5 after infection of recipients in the adoptive transfer system has proven to be the most effective method for preventing the development of a recipient-derived CTL response within 4 days of cell transfer.

Thus, a sensitive assay is available for studying the role of inflammatory cells in LCM. The ability to quantitate the number of cells in CSF allows a far more accurate assessment than is possible with other methods of measuring inflammation, such as footpad swelling. The relevance of the various types of inflammatory cells is also easier to determine, since there are few resident defense cells in either the CSF or brain, and the population of immune cells can be manipulated by the passive transfer system.

3 Cellular Constituents of Meningitis

3.1 Cytotoxic T Cells

The presence of CTLs in the CSF has been shown both by functional studies and by immunocytochemistry. Thy 1^+ cells present in CSF 6–7 days postinfection specifically kill LCMV-infected cells (ZINKERNAGEL and DOHERTY 1973). CTL activity is also present in the CSF 3 days after immune-cell transfer to infected, immunosuppressed animals (ALLAN and DOHERTY 1986). Finally, the

presence of cells bearing the Lyt 2^+ marker, which is characteristic of class I MHC-restricted CTL, has been demonstrated by FACS analysis of the CSF collected 6 days after intracerebral inoculation of LCMV (J.E. DIXON and R. CEREDIG, unpublished results).

3.2 Natural Killer Cells

High levels of natural killer (NK)-cell activity, as judged by lysis of YAC cells, the NK-sensitive murine lymphoma line, can be detected in the CSF and cervical lymph nodes 6–7 days after intraneural infection with LCMV (ALLAN and DOHERTY 1986). However, it appears unlikely that the lytic activity of these cells is essential for the disease process, since removal of NK-cell activity by treatment of mice with antibody to asialo GM1 ganglioside 4 or 5 days after intracerebral inoculation of LCMV failed to alter the development of the disease significantly, although NK-cell activity was substantially eliminated from the spleen and lymph nodes (ALLAN and DOHERTY 1986). Further attempts to analyze the role of these cells by treatment of recipient mice with this antibody prior to passive transfer of immune cells were unsuccessful, since the treatment was found to interfere with the development of CTL activity in the cyclophosphamide-treated animals (P.C. DOHERTY and J.E. ALLAN, submitted). Examination of mutant mice of the C57BL/6 strain, which are homozygous for the beige mutation and therefore deficient in NK-cell activity, showed that they were not significantly different from their NK cell-competent heterozygous littermates in either susceptibility to LCM or in the time of onset of inflammation (ALLAN and DOHERTY 1986). Since 3 days after adoptive transfer of syngeneic immune cells to immunosuppressed, LCMV-infected C57BL/6 recipient mice, cells from the CSF or spleen showed no capacity to kill YAC cells (ALLAN and DOHERTY 1986), it is unlikely that NK cells play an essential role in LCM immunopathology.

3.3 Macrophages

The presence of activated monocytes in the LCM meningeal exudate has been documented by histological studies (SCHWENDEMANN et al. 1983). The occurrence of these cells, and also of resident macrophages, has been studied further by staining brain tissue with F4/80, a monoclonal antibody that has been shown to react specifically with cells of the monocyte/macrophage lineage (AUSTYN and GORDON 1981). Approximately 18% of infiltrating cells were found to be F4/80^+ on day 7 after intraneural inoculation of LCMV (J.E. DIXON and P.C. DOHERTY, unpublished data). Staining for Ia antigen, an inducible class II MHC antigen, suggested that both the resident macrophages and infiltrating monocytes present in the CNS were activated. By the third day after immune-cell transfer to cyclophosphamide-suppressed, virus-infected recipients, a high proportion of the inflammatory cells in the brain were macrophages (45%).

To summarize, macrophages, NK cells, and CTLs have been shown to be present in the meningeal exudate of animals infected with LCMV by the intraneural route. Evidence for the presence of macrophages and CTL, but not NK cells, can also be found in the meningeal infiltrate induced by cell transfer to infected, immunosuppressed recipients.

4 Triggering of Meningitis and Targeting of T Cells

4.1 Requirement for Lyt 2^+ T Cells

Lyt 2^+ T cells seem to be essential for triggering infiltration into the CNS, since the removal of Thy 1^+ or Lyt 2^+ cells, but not L3T4$^+$ cells from the immune population prior to their passive transfer to virus-infected, immunosuppressed recipients prevents the appearance of cells in the CSF 3 days later (Fig. 2). In addition, recipient animals lack histological evidence of cell infiltration into brain tissue on days 1, 2, or 3 after transfer of Lyt 2^+ cell-depleted populations (J.E. DIXON, J.E. ALLAN, P.C. DOHERTY, submitted). The results of a previous study, in which meningitis followed the transfer of cells depleted of the Lyt 2^+ population (ALLAN and DOHERTY 1985a), may be explained by the failure of cyclophosphamide treatment given to recipients before virus infection to prevent generation of host-derived Lyt 2^+ cells. As mentioned above, administration of cyclophosphamide later during infection overcomes this problem (ALLAN and DOHERTY 1985c).

The T cell present in cyclophosphamide-suppressed, LCMV-infected mice after passive transfer of immune cells are of donor and not recipient origin. This has been demonstrated by the transfer of C57BL/6 Ka Thy 1.1^+ immune cells to Thy 1.2^+ C57BL/6 mice. Three days after transfer, cells from recipient

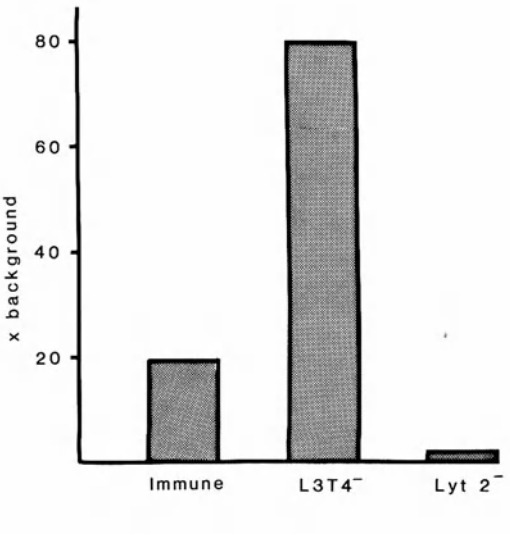

Fig. 2. Number of white blood cells in the CSF as a multiple of the concentration observed in control mice. LCMV-infected, cyclophosphamide-suppressed C57BL/6 mice were given 2×10^7 syngeneic immune cells, which were either untreated or treated with monoclonal antibodies to L3T4, or Lyt 2, and complement. Control mice did not receive cells. The number of cells in the CSF were counted 3 days later in groups of five mice

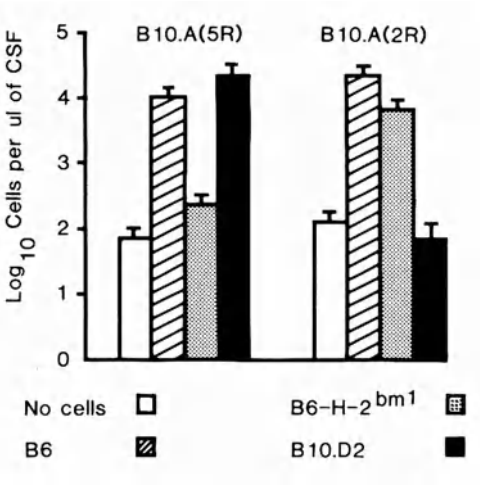

Fig. 3. Effects of class I or class II MHC compatibility between donor immune cells and virus-infected, cyclophosphamide-suppressed recipient mice. Recipient mice cells were B10.A5R (K^b I^b D^d) and B10.A2R (K^k I^k D^d). Donor cells were B6 (K^b I^b D^b), B6-H-2^{bm1} (K^{ba} I^b D^b), and B10.D2 (K^d I^d D^d). Cells were counted from groups of four and five mice and represent the mean ± SE

spleens killed 33% of virus-infected cells at an effector:target ratio of 50:1. This value was not reduced by treatment of cells with Thy 1.2 antibody and complement (41%) immediately before assay. In contrast, only 14% lysis occurred after treatment with antibody to Thy 1.1 and complement, demonstrating that the CTLs in recipient animals were substantially Thy 1.1^+ and thus of donor origin.

4.2 Class I MHC Restriction of Disease Transfer

Severe meningitis ensues in the adoptive transfer model only when there is matching of class I MHC antigens between donor immune cells and recipient mice (Doherty et al. 1976). In comparison, compatibility of class II MHC antigens is of lesser importance (Doherty and Allan 1985). As shown in Fig. 3, class I MHC compatibility at the *D* region gives severe meningitis (B10.D2→5R, B6→5R; bm1→2R), whereas compatibility only for class II MHC antigens (bm1→5R) does not result in a significantly greater number of cells in the CSF than that found in nonreconstituted mice.

4.3 Targeting of T Cells to Neural Tissue

The events that target T cells to the CNS, and also the extent to which the severity of inflammation is determined by neural tissue rather than by lymphoid tissue have received little attention. The observation of a 3- to 4-day delay before the development of severe meningitis following the intravenous transfer of activated immune cells to recipient mice suggests that T cells may proliferate elsewhere in the recipients before entering the CNS (Fig. 4A). An alternative proposal is that T cells travel directly to the CNS, but are not observed until they have proliferated substantially at this site (Fig. 4B) and have attracted other types of cells into the inflammatory lesion.

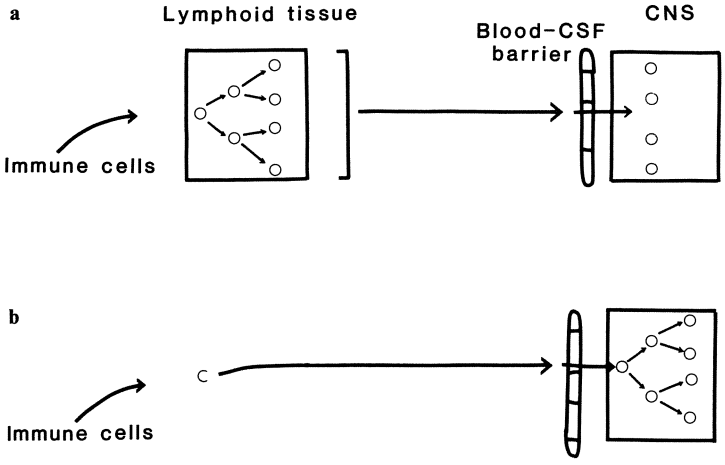

Fig. 4a, b. Proposals for the events that might follow the adoptive transfer of immune cells to LCMV-infected, immunosuppressed mice. In **a** the LCMV-immune cells proliferate in lymphoid tissue before localizing to the CNS. In **b** T cells home to the neural tissue immediately on transfer, proliferate at this site, and enable other cells to enter the CNS

Table 1. An investigation into the requirements for targeting of T cells to the CNS using cyclophosphamide-suppressed chimeric mice as recipients[a] for immune-cell transfer

Assay[b]	Donor cells	[k→k × b]	[b→k × b]	[k × b→k]	[k × b→b]
CTL	k	33	13	20	40
Meningitis		812	138	257	27
CTL	b	60	55	60	40
Meningitis		457	446	4	234

[a] The CBA/H, C57BL/6, and (CBA × B6)F1 recipients were irradiated (950 rads) and reconstituted with bone marrow 24 h later. The chimeras were used as virus-infected, immunosuppressed recipients for immune BALB/c H-2^b and B10.Br T cells, 8–10 weeks later

[b] CTL activity in spleens of mice given WE3 LCMV intravenously. Activity was tested by ^{51}Cr release assay on infected and uninfected cells histocompatible with the donor cells. Results are for 10:1 effector:target ratio. Meningitis was measured as a multiple of background for mean CSF counts from 4 or 5 mice given ARM LCMV intracerebrally, followed by cyclophosphamide and immune spleen cells. The "background" counts were from (k × b)F1 mice that were given virus and cyclophosphamide, but no immune cells

Bone marrow radiation chimeras have been used as infected, immunosuppressed recipients to analyze these events. The chimeras were prepared so that in some cases the MHC phenotype of the donor bone marrow-derived cells differed from that of the radiation-resistant host cells. The most severe inflammation, as judged by the number of cells in the CSF, was observed when the donor immune cells shared at least one MHC haplotype with both the bone marrow donor and the radiation-resistant host component. There was little evidence to support the idea that transferred T cells home directly to the brain

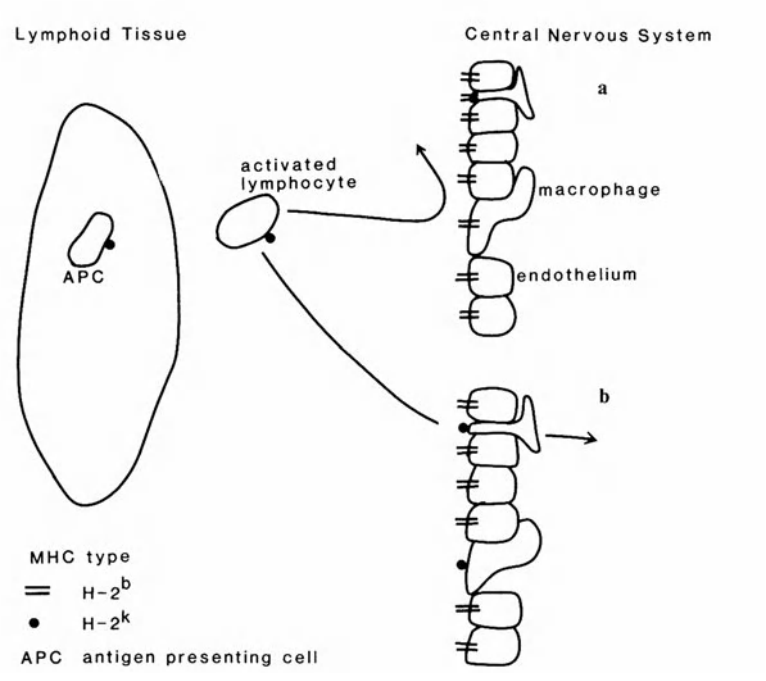

Fig. 5a, b. Mechanisms that may control the entry of T cells into the CNS of chimeric mice. In **a** the cells forming the blood-brain barrier are radiation-resistant cells, which differ in MHC phenotype from the reconstituting bone marrow cells. As a consequence, transferred immune cells of the same MHC type as the bone marrow cells cannot enter the CNS. In **b** some of the macrophages associated with the CNS have been replaced by the progeny of the reconstituting bone marrow. T cells may then be able to enter the neutral tissue if these macrophages present LCMV

since immune cells of C phenotype inoculated into $[(A \times B) \rightarrow (A \times C)]$ recipients. where A, B, and C represent different MHC haplotypes, resulted in 10-fold less inflammation than that induced from A-immune cells given to $[(A \times B) \rightarrow (A \times C)]$ chimeras.

The necessity for the expression of appropriate antigens on cells constituting the blood-CSF barrier was indicated by the observation that the transfer of H-2^b immune cells to $[(k \times b) \rightarrow (b)]$ or to $[(b \times d \rightarrow (k \times d)]$ animals resulted in CTL activity in the spleen without the development of meningitis (Table 1). An apparently contradictory result was the observation of weak, but significant, inflammation following transfer of H-2^k immune cells to $[(k \times b) \rightarrow (b \times d)]$ chimeras. One explanation for these observations is that H-2^k immune T cells may cross-react with H-2^b + LCMV. Alternatively, the infiltrate could consist of cells, such as NK cells, which are not MHC-restricted in activity. However, as described above, there is no evidence to suggest that NK cells are involved in this adoptive transfer model. Another possibility is, as depicted in Fig. 5, that radiation-resistant macrophages associated with the blood-brain barrier may be gradually replaced by cells of donor bone marrow origin, as occurs with antigen-presenting cells in the thymus (Longo and Schwartz 1980). A

difference in the rate of cell turnover between strains of mice could explain the divergence in findings for the sets of chimeras.

It thus appears that, for a maximal inflammatory process to occur, class I MHC-restricted Lyt 2^+ cells proliferate first in the peripheral lymphoid tissue and then enter the brain following recognition of class I MHC-compatible infected cells at the blood-CSF barrier.

5 Influence of MHC Phenotype on Meningitis

5.1 Immune Response Genes

Gene transfection studies have shown that, for mice of the *H-2*^{d} haplotype, the LCMV-specific CTL response is associated only with *H-2L*^{d} and not with *H-2K*^{d} or *H-2*^{d} (ORN et al. 1982). Since the CTL (Lyt 2^+ phenotype) response is essential for the induction of meningitis, it would be expected that the absence of *H-2L*^{d} should decrease the severity of inflammation. The C-H-2^{dm2} mutant strain of BALB/c mice lacks *H-2L*^{d}, and we have found that, following intracerebral inoculation of LCMV, there is a delay of approximately 24 h in the onset of severe inflammation and symptoms. This suggests tht the CTL response is not crucial for the development of LCM. However, a CTL response against LCMV-infected BALB/c cells was induced in virus-primed C-H-2^{dm2} spleen cells by culture with LCMV in the presence of syngeneic stimulator cells. In contrast, restimulated BALB/c cells did not recognize infected C-H-2^{dm2} cells. These observations, in conjunction with the finding that B10.HTG (K^d D^b) and perhaps also B10.5R (K^b D^d L^d) LCMV-immune spleen cells, were able to kill C-H-2^{dm2} cells, show that a K^d (and perhaps D^d) restricted CTL response can occur when there is no *H-2L*^{d}-restricted response. Thus, in the absence of a strong immune response gene (*H-2L*^{d}) for CTL activity, meningitis is delayed, governed by other, weaker (*H-2K*^{d} > *H-2D*^{d}) immune response genes (ALLAN and DOHERTY 1985b). Comparable findings have been made previously for the vaccinia model (DOHERTY and KORNGOLD 1983), although the immunodominance effect is less absolute than that described here.

5.2 Hybrid Resistance to Cell Transfer

Hybrid resistance has been found to influence the severity of meningitis induced by immune cell transfer into LCMV-infected, cyclophosphamide-treated mice (DOHERTY and ALLAN 1986). This phenomenon has previously inhibited the growth of tumor and bone marrow cells that are homozygous for *H-2D*^{b} on transfer to F1 recipient mice which contain the *H-2*^{b} allele and are heterozygous at *H-2D* (reviewed by CLARK and HARMON 1980). However, it is not generally recognized that the function of mature T-cell populations may be inhibited in the same way. B6 (H-2K^{b}D^b) LCMV-immune cells induce less severe meningitis and lower CTL responses in (CBA × B6) F1 or (BALB/c × B6) F1 recipients

Table 2. Hybrid resistance inhibits the development of meningitis following the adoptive transfer of immune cells to LCMV-infected, immunosuppressed mice

Donor strain	Number of spleen cells ($\times 10^7$)	Recipient	Meningitis[a]	Number of spleen cells ($\times 10^7$)	Recipient	Meningitis[a]
B6	1.0	B6	3890	1.0	(BALB/c × B6)F1	30
	0.5		776	0.5		12
(CBA × B6)F1	1.0		2512	1.0		234
	0.5		309	05		59

[a] The severity of meningitis was assessed as a multiple of background, using the criteria described in the notes to Table 1. The background number of CSF cells in recipients that were not given immune spleen cells was 97 cells/μl in the B6 and 170 cells/μl in the (BALB/c × B6)F1

than in B6 recipients (Table 2). The extent of inhibition is dependent on the dose of cells transferred, and on homozygosity for *H-2D*^{b} in the donor together with heterozygosity for *H-2D*^{b} in the recipient. These observations suggest that hybrid resistance operates to inhibit the development of meningitis.

SPRENT and KORNGOLD (1983) have shown that hybrid resistance inhibits T cell recirculation, based on studies using thoracic duct cannulation. It is not known whether LCMV-immune T cells given intravenously need to recirculate from blood to lymph, although the studies with chimeras (see above) indicate that these T cells must replicate in the spleen before entering the CSF. It is thus possible that hybrid resistance impedes both the localization and proliferation of T cells in lymphoid areas, and perhaps also inhibits trafficking from the periphery to the CNS. The potential operation of hybrid resistance should be taken into account in all T-cell transfer systems for which relatively low numbers of donor-immune cells are used.

6 Cellular Basis of Immunopathology

The previous sections have presented strong evidence for the concept that 2^+ T cells trigger meningeal inflammation. Presumably meningitis does not occur before the development of the T-cell response because the nonspecific NK cells, which are activated within a few days of infection, do not recognize the infected choriomeninges (see WELSH, this volume) and this relatively nonlytic virus, itself, is unlikely to disrupt the blood-brain barrier (DOHERTY and ZINKERNAGEL 1974). It has not been made clear whether the role of the Lyt 2^+ population is: (a) simply to initiate meningitis through destruction of the blood-brain barrier, (b) in addition, to sustain the infiltration of cells into the CNS, or (c) to produce pathology directly by lysis of virus-infected neural cells. The actual clinical reasons for death are not known, a situation that has not altered greatly since they were discussed by DOHERTY and ZINKERNAGEL (1974). It ap-

pears from electron microscopy that the level of cytopathology is insufficient to explain the lethal outcome of the disease (WALKER et al. 1975; SCHWENDEMANN et al. 1983).

The outcome of meningitis seems to depend on a balance between the intensity of the immune response and the extent of LCMV replication in nervous tissue (reviewed by NATHANSON et al. 1975; THOMSEN et al. 1979). Both protection from and induction of fatal disease have been shown to be mediated by the Lyt 2^+ population in adoptive transfer studies (ALLAN and DOHERTY 1985a). Recipients injected with large amounts of virus showed greater inflammation within 3 days of immune-cell transfer, clearance of virus from the brain, and a reduction in mortality rate in comparison to recipients given a 10-fold lower dose of virus. This lower dose of virus resulted in less inflammation during the early period after cell transfer and a greater mortality rate. Removal of L3T4$^+$ cells from the transferred population did not affect these observations; both the protective effect and the fatal meningitis required Lyt 2^+ cells (ALLAN and DOHERTY 1985a).

Confirmation that the Lyt 2^+ T cells are protective comes from two studies with cloned LCMV-specific T-cells lines. BYRNE and OLDSTONE (1984) found that intravenous inoculation of these cells cleared LCMV from the spleen within 24 h, and BAENZIGER and colleagues (1986) showed that lethal LCM can be prevented by intracerebral inoculation of cloned T cells together with virus.

The immunopathological nature of the activity of Lyt 2^+ cells has been most clearly demonstrated by the observation that intracerebral inoculation of cloned Lyt 2^+ cells into acutely infected, irradiated, or cyclophosphamide-treated mice resulted in death 3–5 days later (BAENZIGER et al. 1986). These studies suggest that Lyt 2^+ cells act directly rather than through the recruitment of circulating monocytes. BAENZIGER and colleagues (1986) have commented that, according to their unpublished observations, fatal disease induced by LCMV occurs in the absence of severe inflammation when cloned LCMV-specific Lyt 2^+ lines are injected intracerebrally into mice irradiated previously with 750–900 rads. In this system, the only other cells that can participate are radiation-resistant cells within the CNS. This possibility cannot be dismissed, since cells of the monocyte/macrophage lineage have been identified in neural tissue (PERRY et al. 1985).

It would appear, therefore, that many of the cells which form the meningeal exudate must enter nonspecifically. This has already been described for cells in the CSF during vaccinia virus-induced meningitis, in which almost 1% of Lyt 2^+ cells were found to be specific for the influenza virus with which the mice had been immunized more than 1 month previously (HURWITZ et al. 1983).

7 Summary and Conclusions

Several types of cells contribute substantially to inflammation: those considered include Lyt 2^+ T cells, NK cells, and macrophages. The models used to analyze the roles of these cells are summarized in Table 3. Other types of cells known

Table 3. Summary of the evidence for the presence of various cell types in LCM and of procedures used to examine their role in the development of meningitis

Cell type	Model[a]	Procedure
NK cells	Direct	Lytic activity against YAC cells in CSF; $ASGM_1$[b] treatment ineffective; bg/bg mutation ineffective
	Indirect	No activity in CSF
Macrophages	Direct	$F4/80^+$ cells in brain
	Indirect	$F4/80^+$ cells in brain
Cytotoxic T cells	Direct	Presence of Lyt 2^+ cells in CSF; LCMV-specific CTL in CSF; delayed meningitis in C-H-2^{dm2} mice
	Indirect	Lyt 2^+ cells required for meningitis; class I MHC-restricted disease transfer; intracerebral inoculation of cloned Lyt 2^+ cells induce LCM

[a] *Direct*, intracerebral inoculation with LCMV; *indirect* adoptive transfer of immune spleen cells to infected, immunosuppressed mice

[b] $ASGM_1$, antibody to asialo GM_1 ganglioside

to be present (but not discussed here) are the L3T4 subset of T cells (R. CEREDIG, J.E. ALLAN, P.C. DOHERTY, submitted), neutrophils (which are relatively rare), and also B cells, which have been shown by treatment of animals with anti-mu serum not to be essential for the disease process (JOHNSON et al. 1978).

Class I MHC-restricted Lyt 2^+ T cells are the only cell population found to be essential for the development of fatal LCM. They are responsible for the initiation of meningitis and also apparently for the changes that lead to the development of neurological disease. Cell transfer systems can be manipulated to result in either death or recovery depending on the dose of virus used and the timing of administration of immune T cells. The severity of meningitis has been found to be influenced by genetic control over the immune response, and also by the hybrid histocompatibility effect in adoptive transfer systems.

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Clearance of Virus by T Lymphocytes Mediating Delayed Type Hypersensitivity

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1 Historical Trends in the Study of Delayed Type Hypersensitivity

The mammalian defense system against invading microorganisms has been investigated for more than a hundred years, with the ultimate goal, therapeutically and prophylactically, to overcome human disease caused by infectious agents. Not surprisingly, at present such experimentation focuses considerably on viruses, since these agents do not to any noteworthy degree respond to antibiotics or chemotherapy. In spite of this research, however, it is still uncertain by which mechanisms exactly viruses are cleared from the infected host, and how virus-induced, cell-mediated pathology develops. Although the phenomenon of delayed type hypersensitivity (DTH) – of all the immunologically specific mechanisms believed to be important in virus elimination – was the first to be discovered, it is still far from fully understood.

As early as the last decades of the eighteenth century, Edward Jenner noticed that people who had previously suffered from small pox or cow pox, or had been vaccinated with the "cow pox" (vaccinia) virus, did not develop vesiculopostular lesions, subsequent to cutaneous insertion of vaccinia or variola, but

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showed only a red swelling around the site of inoculation (JENNER 1889). Furthermore, this erysipelatous efflorescence appeared and disappeared more rapidly than did the lesions following primary infection with either of the two viruses. Having observed this "disposition to sudden cuticular inflammation," Jenner reflected that this phenomenon "becomes almost a criterion by which we can determine whether the infection will be received or not.... It seems as if a change which endures through life had been produced."

About one hundred years later Robert Koch, studying experimental tuberculous infection, observed that inoculation of bacilli (or extracts thereof) into tuberculous guinea pigs resulted in the appearance of an inflamed area after 24–48 h, although little or no reaction occurred in normal guinea pigs until 10–14 days after an analogous infection (KOCH 1890). This "Koch phenomenon" became the prototype for dermal skin tests (e.g. DTH) used in many infectious diseases to monitor the cell-mediated immune status of the patient.

This change of state, reflected in the ability of an organism to raise an accelerated response to foreign matter, somewhat reminiscent of the anaphylactic reaction, coupled with the delay in the appearance of the cutaneous reaction as compared to anaphylaxis, gave the phenomenon its name. When it was later shown that such a delayed type of reactivity could be induced by many different proteins, the fundamental difference between hypersensitivity of the immediate and delayed types was strongly underlined by the demonstration that contact sensitivity and sensitivity to intracellular parasites could be transferred by cells only, but not by antibodies (LANDSTEINER and CHASE 1942; CHASE 1945; WESSLEN 1952; COE et al. 1966).

Much detailed and fundamental information on the DTH reaction has been obtained through studies of hapten-carrier systems. However, it is generally believed that DTH to nonmicrobial antigens is the same phenomenologically as that to microbial proteins (DAVID 1968).

The cutaneous inflammation of the tuberculin type is considered the typical manifestation of DTH and is thus a characteristic secondary cell-mediated immune response, but its histopathology is also dictated by the special anatomical features of the skin. Therefore the importance of the morphology of the lesion should not be overexaggerated when considering the biological role of DTH in experimental infections. In addition, the pathology of the local lesion has been seen to differ with the sensitizing antigen and may vary in severity from cellular infiltration to massive necrosis (WAKSMAN 1960). In the context of antimicrobial DTH it might also be of importance to remember that the classical reddening and swelling of the skin around the site of antigen injection is not the only manifestation of DTH. In hypersensitive tuberculous patients, a systemic reaction occurs if sufficient quantities of tuberculin gain access to the blood stream (STETSON 1959). This leads to symptoms including fever, malaise, backache, pains in the joints, and, in severe cases, shock and death. Similar reactions can be observed in experimental animals sensitized to hapten-protein conjugates (SALVIN 1962).

On the other hand, the local DTH reaction has important characteristic features, of which the most basic, perhaps, is the fact that it depends fundamentally on two cellular elements present in the inflammatory exudate. As many

as 90% of the accumulated cells have been shown to be uncommitted mononuclear cells, derived from rapidly dividing precursors in the bone marrow (McCluskey et al. 1963). The cells responsible for the passive transfer of DTH are specifically committed lymphocytes, which may belong to at least two different subsets of the T-cell population (Liew 1982). Both monocytes and T cells are mandatory for the elicitation of DTH, and both are known to secrete physiologically active soluble mediators (Klein 1982).

Although massive tissue destruction as seen in tuberculosis was connected with DTH very early on, the question of whether DTH represents an immunological mechanism by which elimination of microorganisms is facilitated has been subject to lively debate for many years, and yet the accumulated data seem inconclusive. However, many findings support the idea of an intimate relationship between DTH and cellular resistance to infection. For example, in experimental tuberculosis, the concomitant appearance of dermal hypersensitivity and a drop in the number of bacilli has been demonstrated (David 1968). Furthermore, when the hypersensitive state is eliminated by desensitization, the tuberculous lesions in guinea pigs show neither a decrease in the number of bacilli nor necrosis (David 1968). More recent observations have also substantiated this view. Thus, well-defined antigen-specific T-cell populations and T-cell clones have been shown to represent cell types crucially involved in protection against infection with *Listeria monocytogenes* and also in DTH to listerial antigens (Kaufmann et al. 1979b; Kaufmann and Hahn 1982).

One obstacle in the investigation of the role of the DTH reaction in infectious diseases is the lack of a sensitive and precise assay system. In rodents the footpad test is still the most widely used technique.

With the demonstration of virus-specific cytotoxic T cells (Marker and Volkert 1973; Doherty et al. 1974), the effect of which was easily and objectively determined in vitro, the study of cell-mediated immunity in microbial infections entered a new era, and the biological role of this T-cell subset is still considered to be central. On the other hand, in experimental virus infections, for which some emphasis has again been laid on DTH, striking examples of concordance between virus clearance and/or immune-mediated pathology on the one hand and the capability of the animals to mount a virus-specific DTH reaction on the other have been demonstrated (Nash and Gell 1981; Liew 1983; Thomsen et al. 1983a, b; Marker and Thomsen 1986; Thomsen and Marker 1986). Nevertheless, direct proof is still scarce, as has been the case in historical discussions on immunity to tuberculosis. Circumstantial evidence, some recently obtained, is presented below.

2 DTH as an Expression of T-Effector Function

2.1 Development and Quantitation of DTH

In the classification of hypersensitivity suggested by Gell and Coombs in 1963, DTH (type IV hypersensitivity) was used as a category to cover all hypersensitivity reactions that took more than 12 h to develop (Gell and Coombs 1963).

Today DTH could probably more correctly be defined as an inflammatory reaction that is immunologically specific, peaks after 24–48 h, and can be transferred with sensitized, lymphokine-producing T cells. The histological appearance is characterized by localized edema and cell infiltration dominated by mononuclear cells (DIENES and MALLORY 1932).

Cellular mediation of DTH was first demonstrated by transfer of sensitivity using unfractionated cells from peritoneal exudates (LANDSTEINER and CHASE 1942; CHASE 1945), and recently it has proved possible to transfer DTH with cloned T cells (BIANCHI et al. 1981; LIN and ASKONAS 1981). However, antigen-specific T cells are not the final mediators of DTH but rather initiators of a cascade that involves accumulation and activation of other cells. In fact, the majority of cells participating in DTH are uncommitted (MCCLUSKEY et al. 1963), which is underscored by the finding that a single T cell may be sufficient to bring about gross expression of DTH (MARCHAL et al. 1982). The recruited nonspecific cells are essential to the development of the DTH reaction, since preirradiation of recipients of primed T-cell grafts abolishes not only the cell infiltration but also the erythema and edema typical of DTH at the macroscopic level (COE et al. 1966). Bone marrow cells from unsensitized donors can make good the deficit created by irradiation (LUBAROFF and WAKSMAN 1968). Since the radiation-induced defect may also be overcome by injecting the test site with uncommitted peritoneal exudate cells (VOLKMAN and COLLINS 1971), it is generally accepted that cells of the monocyte-macrophage lineage constitute the crucial population. However, there is no direct proportionality between cell infiltration and gross manifestation of DTH (like footpad swelling), and only a small number of newly formed migratory cells are required for expression of DTH (VOLKMAN and COLLINS 1968). The presence of large numbers of macrophages in the DTH lesion has been confirmed by use of modern immunohistochemical methods (PLATT et al. 1983), but the precise mechanism underlying the role of macrophages in the expression of DTH is still not known. It would seem likely, however, that the marked increase in secretion of proteases and reactive oxygen metabolites noted in activated macrophages (DAVIES and ALLISON 1976; NATHAN et al. 1980; KLEBANOFF 1982) may underlie not only the antimicrobial activity but also constitute the basis for the inflammatory reaction and tissue damage often associated with DTH.

Mononuclear phagocytes are not, however, the only population to be attracted to the site of a DTH reaction. Independent of their specificity T-cell lymphoblasts have intrinsic affinity for foci of inflammatory reaction (KOSTER and MCGREGOR 1970; MCCLUSKEY and WERDELIN 1971; ASHERSON and ALLWOOD 1972; NORTH and SPITALNY 1974), and even recirculating memory T cells with irrelevant specificities seem to invade an inflammatory lesion (HURWITZ et al. 1983). In contrast, very few B cells are found in the typical DTH reaction site (PLATT et al. 1983). However, in virus-induced inflammation, B cells may enter in the later phases, appearing first in the perivascular cuffs, then migrating into the tissue (ESIRI 1980; OWENS et al. 1981; MOENCH and GRIFFIN 1984). Polymorphonuclear leukocytes may also be seen in the infiltrate, especially in the very early phase (BLANDEN 1974; TURK 1975; ASKENASE and ATWOOD 1976), but are usually a minor component rather than the dominating cell type, as

is the case for the Arthus reaction. In this respect, the Jones-Mote type of DTH constitutes an exception in that basophilic granulocytes make up a significant part (up to about 50%) of the cellular infiltrate (RICHERSON et al. 1969; KATZ et al. 1974; TURK 1975).

An important question relating not only to DTH but to cell-mediated immunity in general is how the effector cascade is initiated at the relevant site. There is reason to believe that newly formed effector T cells leave their production site and appear in the blood and lymph (BLANDEN 1974). The exact mechanisms by which circulating antigen-reactive T cells find the antigen-containing tissue are unclear, but it seems that there are both nonspecific and specific mechanisms operating in the accumulation of specific T cells at relevant sites.

First, it would appear that a few specific T cells, while randomly monitoring the tissues, could reach the challenge site and after contact with the relevant antigen remain in that location and initiate the inflammation. Development of the DTH reaction is greatly enhanced, however, if the site of antigen challenge is itself intrinsically inflammatory. Thus, it should be remembered that an important feature of newly activated T-lymphoblasts is their propensity to enter inflammatory exudates. Furthermore, there is experimental evidence indicating that a second T-cell subset – in addition to the actual effector Td (DTH-mediating) cells – may serve to direct the immune surveillance to sites of antigen challenge. The mechanism involved seems to be the release of a soluble antigen-specific factor that sensitizes tissue mast cells (ASKENASE and VAN LOVEREN 1983). Upon antigen challenge, monoamines are released from these sensitized mast cells, causing gaps to form between the endothelial cells of the postcapillary venules; in this way antigen-specific Td cells could preferentially enter the reaction site and interact with the antigen.

The specific mechanism depends upon the recognition by antigen-specific T cells of antigen, presumably on the surface of endothelial cells lining the vasculature adjacent to the test site (BURGER and VETTO 1982). This possibility is suggested by the finding that injected radiolabeled effector cells under certain conditions show specific accumulation at sites of antigen challenge (SCHEPER et al. 1985), and also by the classical observation that lymphocytes tend to attach to endothelial cells prior to entering the antigen-containing tissue (DVORAK 1974). Recent studies in vitro support the idea that endothelial cells may function as antigen-presenting cells (HIRSCHBERG et al. 1981).

Once the antigen-specific T cells have entered the extravascular tissue there is little doubt that their interaction with antigen triggers the release of factors which attract, immobilize, and activate mononuclear phagocytes (LIEW 1982; KLEIN 1982), which population constitutes the second major component of the cellular infiltrate associated with a DTH reaction.

It should be clear by now that the DTH reaction is a biological cascade resulting from complex cellular interactions and in the above we have not even considered the regulatory role played by various T suppressor (Ts) subsets. This complexity is reflected in the many methods that have been designed to evaluate DTH responses. In vivo these include: disappearance of macrophages from the peritoneal cavity following antigen challenge (NELSON and BOYDEN 1963), skin thickening, tissue damage, vascular changes, and cell proliferation at the

skin test site (RUDDLE 1972). The footpad or earswelling test devised as early as 1955 (GRAY and JENNINGS 1955) is probably the most widely used for measuring DTH responses in vivo. However, this test is not completely objective, since it measures only the gross manifestation of DTH, thus, it is difficult to determine whether swelling results from cellular infiltration and/or edema. Furthermore, large numbers of cells must be transferred to obtain clear results in adoptive transfer experiments. A radioisotopic assay has been introduced to measure DTH more objectively (SABOLOVIC et al. 1972). This test is based on the observation that replicating cells (labeled by ^{125}IUdR) from the blood infiltrate the test site. However, this assay is no more sensitive than the swelling tests (ROBINSON and NAYSMITH 1976), and from a practical viewpoint, an obvious disadvantage is that the same mouse cannot be followed a certain time span. Moreover, radioisotopes are needed.

Since the DTH reaction can be conceived as the net result of the actions of various lymphokines, it might seem natural to measure these directly. Most frequently used is the migration inhibition test, which measures the inhibition of macrophage migration from capillary tubes in the presence of migration inhibition factor (MIF) (GEORGE and VAUGHAN 1962). Possibly other lymphokines might be more suitable, but as they all belong to the group of inflammatory substances released in response to mild trauma or lymphocyte stimulation, a tight correlation with the in vivo reaction is never likely to be found. Finally, it should be mentioned that lymphocyte transformation has often been used uncritically as an in vitro correlate of DTH. At the clonal level, there seems to be a correlation between the ability of T cells to produce interleukin 2 (Il-2) and to mediate DTH (VON BOEHMER et al. 1984), which might constitute a basis for some correlation between lymphocyte proliferation and DTH reactivity; however, that simple [^{3}H]thymidine uptake in bulk culture could be used to evaluate DTH is unlikely. For instance, in mice tolerized to herpes virus, proliferation can be found in vitro, but no DTH response is seen (NASH and ASHFORD 1982). Similarly, we have found proliferating cells in spleen and lymph nodes of mice infected with a relatively high dose of lymphocytic choriomeningitis virus (LCMV), which suppresses the induction of DTH (unpublished results).

2.2 MHC Restriction and Lyt Phenotypes of Virus-Specific Td Cells

It is a well-established fact that T cells can be activated only by recognizing antigen together with cell membrane molecules coded for by the major histocompatibility complex (MHC restriction). Helper and proliferating T cells recognize primarily class II MHC antigens (*I* region in the mouse) (ERB and FELDMANN 1975; KATZ and BENACERRAF 1975), whereas cytotoxic T (Tc) cells and their precursors recognize predominantly class I antigens (*K, D* and *L* regions in the mouse) (BEVAN 1975; GORDON et al. 1976; DOHERTY et al. 1976; SHEARER et al. 1976; BIDDISON et al. 1978). An extension of these in vitro studies showed that the MHC restriction of T cells function also applies to in vivo reactions, like DTH. This was first shown by MILLER and coworkers (1975), who demonstrated that transfer of DTH to fowl gamma globulin could be accomplished

only when the donor and recipient shared class II but not class I loci. Although ZINKERNAGEL (1976) shortly afterward showed that DTH to LCMV was restricted predominantly, if not exclusively, by class I antigen, thus demonstrating that Td function could be restricted by class I products, it was the dogma for quite a while that Tc and Td cells were fundamentally different in that Td cells were class II restricted, whereas Tc cells were class I restricted. This dualistic perception of the effector arm of the T cell system was further strengthened by the subsequent observation made by Cantor, Boyse, and colleagues that DTH (to sheep red blood cells) was mediated by cells with an Lyt 1^+2^- phenotype – and thus similar to helper (Th) cells – whereas (alloreactive) Tc cells were Lyt 1^-2^+ (CANTOR and BOYSE 1975a, b; HUBER et al. 1976).

Therefore, for a period the LCMV system was a notable and unexplained exception to the idea of Tc and Td cells constituting clearly separable subpopulations. However, data obtained in the murine influenza and Sendai virus models may now offer an explanation for this phenomenon. In these models inactivated influenza or F^- Sendai virus induces I region-restricted Lyt 1^+2^- Td cells, whereas infectious influenza virus or F^+ Sendai virus induces an additional *K, D* region-restricted Lyt 1^-2^+ Td subset (ADA et al. 1981). When the inductive requirements for Td effector cells were studied in detail in vitro it could be clearly demonstrated that the selection of the responding T-cell subset(s) reflected the conditions of antigen presentation (ERTL 1981). If noninfectious but F^+ Sendi virus was used, spleen lymphocytes, tumor cells, and LPS blasts could function as antigen-presenting cells to induce selectively the *K, D* region-restricted response. In contrast, noninfectious F^- virus selectively induced the *I* region-restricted subset, and this induction required adherent cells for effective stimulation to occur. Other Ia^+ cells like LPS blasts were ineffective in the presentation of noninfectious F^- virus. The conclusions to be drawn from these experiments are: (a) both Lyt 2^+ and Lyt 2^-, class I- and class II-restricted T cells may function as Td effectors and that it is the mode of antigen presentation which determines whether a given Td-effector subset will develop; (b) apparently any permissive cell may function to present infectious virus in a *K, D* region-restricted fashion; and (c) noninfectious virus (without fusion capacity) is treated by the immune system as inert particles that require processing and presentation by classical Ia^+ antigen-presenting cells for induction to occur. The pattern of the DTH response to a given virus infection, therefore, presumably depends on whether or not permissive replication provides the major antigen stimulus (ADA et al. 1981; NASH 1984). If the former is the case, as is likely in LCMV infection during which virus spreads and replicates rapidly throughout the body, a *K, D* region-restricted response will previal, in particular since such conditions also favor the induction of Ts cells that inhibit the generation of *I* region-restricted Td cells (LIEW and RUSSEL 1980; ADA et al. 1981). The opposite situation is probably exemplified by murine herpes infection, in which the DTH response is mediated solely by *I* region-restricted, Lyt 2^- Td cells (NASH et al. 1981b; NASH and GELL 1983). This virus apparently does not replicate in the lymphoid system, and the bulk of viral antigen is therefore presented to the immune system by conventional Ia^+ antigen-presenting cells.

The fact that both Lyt 2^- helper-like T cells and Lyt 2^+ Tc-like cells may mediate DTH immediately raises the question of identity between T-cell subsets expressing identical markers. The use of long-term clones has established that T cells may be capable of exerting more than one function; Lyt 2^- cells may thus mediate both DTH and help for the antibody response (DENNERT et al. 1981; KAUFMANN and HAHN 1982); similarly, Lyt 2^+ cells may be cytotoxic in vitro and also cause DTH in vivo (LIN and ASKONAS 1981). This appears to hold true also in the LCMV system (BAENZIEGER et al. 1986). However, whether such multifunctional long-term clones are relevant to the in vivo situation where regulatory mechanisms may act to maintain monofunction is difficult to say. Probably the best answer available comes from a study by VON BOEHMER et al. (1984), who showed that about one-third of Tc cell clones to a variety of antigens temporarily also express DTH, thus suggesting that, with regard to the Lyt 2^+ subset, DTH is a function expressed by some but not all Tc cells, and perhaps is mediated by cells in a particular differentiation state.

It is noteworthy that although primary Td effectors to LCMV are *K, D* region-restricted and Lyt 2^+, ALLAN and DOHERTY (1985) studying cell infiltration in the cerebrospinal fluid of animals infected intracerebrally (i.c.), which would seem to be an expression of DTH, also found a Lyt 2^-, class I-restricted effector subset. It should also be mentioned that Pfau and coworkers found a predominant Lyt 2^- subset that appeared after restimulation in vitro with infectious virus (PEVEAR and PFAU 1984). Thus, perhaps also in the LCMV model the experimental situation may determine the effector subset revealed.

It is clearly established that the transfer of DTH is restricted by the *H-2* complex. However, since the success of in vivo transfer depends on much more than mere recognition by the T cells of the proper antigenic structure, it would not seem unreasonable if additional barriers existed. It is therefore of interest that BERCHE and NORTH (1982) found marked restriction of Listeria-specific DTH when reciprocal transfer was attempted between *H-2* compatible mouse strains differing at non-*H-2* loci. In fact, restriction was as pronounced as that observed when transfer was attempted across the *H-2* barrier. In a series of experiments we tested the ability to transfer LCMV-specific DTH reciprocally between *H-2* compatible strains of mice differing in their non-*H-2* background. We found (unpublished results) that transfer of DTH was always possible between mice sharing the *H-2* haplotype (Table 1). In about half the transfers, syngeneic recipients did give a better response. In the other half, however, allogeneic recipients tended to present the largest DTH reaction. Notably the general pattern was that mice of one strain functioned as the better recipients for both donor strains involved in the experiment, and also that cells from one strain induced the better response in both groups of recipients. Thus, in the LCMV system, we cannot find the same strict requirements for non-*H-2* sharing, that have been reported in the Listeria model. It is conceivable, however, that experiments with strictly defined variants of LCMV would give a different result.

While the importance of Ts in the regulation of the DTH response will be dealt with later, it seems relevant at this point to comment on the involvement of Th in the induction of Td effectors. Antigen-specific Th cells have been

Table 1. Passive transfer of lymphocytic choriomeningitis virus-specific delayed type hypersensitivity between *H-2* compatible strains not sharing the non-*H-2* background

Experiment No.	Mouse strains				Increase in footpad thickness at time after cell transfer	
	Donor		Recipient		24 h	48 h
1 ($H\text{-}2^b$)[e]	C57BL/6	(30)[a]	C57BL/6	(5)[b]	26(10–28)[c]	59(48–62)[d]
			C3H.SW	(4)	19 (5–23)	36(29–43)
	C3H.SW	(100)	C57BL/6	(4)	49(34–66)	85(69–106)
			C3H.SW	(4)	38(25–48)	58(55–75)
2 ($H\text{-}2^k$)	C3H/Ssc1	(40)	C3H/Ssc1	(5)	16 (5–19)	17(12–26)
			CBA/J	(5)	23(15–36)	24(17–34)
	CBA/J	(100)	C3H/Scc1	(5)	17 (6–20)	32(31–43)
			CBA/J	(5)	41(32–43)[d]	60(51–68)[d]
3 ($H\text{-}2^b$)	C57BL/10	(100)	C57BL/10	(4)	28(22–24)	61(52–79)
			Balb.B10	(5)	42(34–47)	71(45–95)
	Balb.B10	(90)	C57BL/10	(3)	8 (7–13)	48(38–68)
			Balb.B10	(4)	22(20–22)	55(40–72)
4 ($H\text{-}2^k$)	C3H/HeJ	(100)	C3H/HeJ	(5)	18(16–36)	50(33–64)
			AKR/N	(5)	12 (6–35)	51(33–85)
	AKR/N	(60)	C3H/HeJ	(5)	7 (4–11)	13(11–17)
			AKR/N	(4)	13 (8–31)	32(15–33)[d]
5 ($H\text{-}2^k$)	CBA/J	(100)	CBA/J	(5)	71(57–79)[d]	77(71–92)
			B10.BR	(5)	17(10–25)	81(68–110)
	B10.BR	(30)	CBA/J	(6)	21(17–25)	28(13–42)
			B10.BR	(3)	7 (5–9)	26(25–26)

[a] Cytotoxic activity ($LU_{50}/10^6$ cells) relative to the higher responding strain
[b] Number of recipients used
[c] Median (and range) of groups; 10^{-2} mm
[d] $P < 0.05$; Mann-Whitney rank test vs allogeneic recipients
[e] *H-2* haplotype of donors and recipients in the experiment

demonstrated both in vitro and in vivo, and the models used involve viral systems, such as influenza and herpes (LEUNG and ADA 1981; TUCKER and BRETSCHER 1982; LIEW 1983). Indeed, in the influenza model the helper cells appear to act in the classical "carrier hapten" associative recognition fashion; mice primed with matrix protein raise an enhanced response to hemagglutinin only if the booster antigen consists of intact whole virus particles (LIEW 1983). This result, however, might seem to conflict with recent experiments indicating that Th cells act through the release of Il-2, since this lymphokine may substitute for their activity (LEUNG et al. 1982). It might be speculated, though, that two Th populations exist, only one of which requires linked recognition. When it comes to T-B collaboration, this latter appears to be the case (TADA et al. 1978; OKUMURA et al. 1979; ADORINI et al. 1983). A common feature of Th cells is a Lyt 1^+2^- phenotype and restriction in induction and expression by the *I-A* subregion of *H-2*. However, the relationship among Th cells assisting

different types of immune responses (antibody, DTH , and cytotoxicity) is not clear at present; available data indicate that at least under certain conditions a single Th cell may help in more than one type of respone (MOLL et al. 1985). It should be mentioned that Th cells for the DTH response have not as yet been found in the LCMV system, probably because transfer experiments in this model are nearly always clouded by the cotransfer of virus.

3 Role of DTH in Antiviral Immunity

3.1 LCMV Model

The murine LCMV infection is perhaps the most intensively studied experimental virus infection and certainly the most well-elucidated arenavirus infection. Studies of this model system have contributed several original and important findings regarding the interaction between an infecting virus and the infected host such as antibody-mediated immune complex disease in otherwise unresponsive virus carriers (OLDSTONE and DIXON 1971 a), the crucial involvement of T cells in fatal choriomeningitis following an i.c. virus inoculation (COLE et al. 1972), virus elimination (VOLKERT et al. 1974), hemopoietic suppression during acute infection (BRO-JØRGENSEN 1978), the demonstration of virus-specific Tc cells (MARKER and VOLKERT 1973; DOHERTY et al. 1974), the necessity of MHC compatibility between the Tc cell and the target (ZINKERNAGEL and DOHERTY 1974), and also the isolation and characterization of LCMV-specific T-cell clones with antiviral activity in vivo when transferred i.v. (BYRNE and OLDSTONE 1984) or, when injected i.c., with the ability to induce classical LCM disease (BAENZIEGER et al. 1986).

Perhaps the earliest significant findings in this model were: (a) the existence of a naturally occurring, often symptomless virus carrier state in mice (TRAUB 1935) – a state that is easily reproduced artificially (VOLKERT 1962), and (b) the observation that large doses of virus inoculated into chicken embryos cause less severe disease than inoculation of smaller doses (BENGTSON and WOOLEY 1936). This seemingly paradoxical phenomenon also applies to mice and was considered in complete accordance with the classical idea of high-dose tolerance (KLEIN 1982).

However, since this theory does not provide any functional explanation of how this tolerant state is established, and since T-cell functions are fundamentally involved in the most critical interactions between LCMV and the infected murine host, this so-called autointerference attracted some attention in several laboratories (HOTCHIN 1971). Thus, LEHMANN-GRUBE and coworkers found pronounced suppression of the primary footpad response and virus-specific Tc activity following inoculation of very high virus doses, which indicated a tolerance of the Td as well as of the Tc function (LEHMANN-GRUBE et al. 1982). This finding was somewhat contradicted by results obtained by PFAU et al., who found equally strong, virus-specific, T-cell-mediated cytotoxicity in spleen cells of mice receiving high and low doses of virus. Since these authors were

Table 2. Mortality in C3H mice after i.c. inoculation with increasing doses of LCMV (reprinted with permission from the *Scandinavian Journal of Immunology* (1985) 21:81–91)

Virus dose (LD_{50})	Number of mice (dead/total)	Death occurred after[a] (days)
Saline	0/10	
10^2	10/10	8.3
10^3	5/10	9.0
10^4	1/10	11.0

[a] Mean time to death among lethally infected mice

also able to demonstrate higher levels of virus in the organs of mice inoculated with large amounts of virus than in those of mice given small amounts, they explained the autointerference by assuming that large numbers of virus-specific T cells are diverted to heavily infected peripheral organs in highly infected mice, thus leaving sensitive structures in the brain undamaged – the "antigen sink" hypothesis (PFAU et al. 1982).

In our latest efforts we have concentrated on experiments in which we used different virus doses as the only immune-modulating intervention. In this model C3H mice were, in nearly all experiments, inoculated with either 10^2 LD_{50} (low-dose mice/animals) or 10^4 LD_{50} LCMV (high-dose mice/animals). Inoculation i.c. was used when immune-mediated pathology was studied, whereas virus was given i.v. in experiments designed to provide information on virus clearance.

Employing this model, we found that i.c. inoculation of 10^2, 10^3, and 10^4 LD_{50} resulted in mortalities of 100%, 50%, and 10%, respectively (Table 2). With the use of 10^4 LD_{50} injected i.v., spleen LCMV titers were found to be $10^{3.0}$ LD_{50}/3 mg tissue 4 weeks after virus inoculation, while little or no virus could be demonstrated at this time in the spleens of mice similarly injected with 10^2 LD_{50}. Thus, it seems clear that our mouse/virus strain combination (C3HSscl/TRAUB strain) constitutes a sensitive system for studying the effect of varying virus doses on reactivity of the immune system.

3.2 Correlation Between Virus-Specific DTH and Clearance

That T cells often play an indispensable role as effector cells in immune-mediated clearance of a primary viral infection is well-known. However, the involved mechanism(s) is not completely understood. Both Lyt 2^+ and Lyt 2^- T cells have been shown to have virus-clearing effects in vivo (YAP et al. 1978; LARSEN et al. 1983; NASH and GELL 1983), and this has been confirmed using cloned T cells (LIN and ASKONAS 1981; SETHI et al. 1983; BYRNE and OLDSTONE 1984; LEUNG et al. 1984).

With regard to the antiviral function of the noncytolytic, class II-restricted, Lyt 2^- subset, herpes simplex virus (HSV)-1 infection is most informative. In

the well-characterized mouse ear model the rapid elimination of infective virus from the ear of an acutely infected animal correlates with the appearance of DTH, and the Td cells share the Lyt 1^+2^- phenotype with the antiviral effectors (NASH and GELL 1983). Furthermore, Ts cells which suppress the Td response also regulate the induction of protective cells (SCHRIER et al. 1985). Therefore, Td effectors were thought to mediate virus clearance through attraction and activation of macrophages and, the DTH reaction was considered the inevitable manifestation of this interaction. However, analysis of an HSV-1-specific, Lyt 1^+2^- clone suggests further complexity in this model; namely, although this clone had a protective effect in vivo and did release macrophage activating factor (MAF) in vitro, no local DTH response was seen (LEUNG et al. 1984). Thus, although the basic scheme for virus clearance outlined above may be correct, the DTH reaction seems to constitute a separate entity, and it has been speculated that the critical role of Td cells lies in the recruitment of relevant effector T cells.

More pertinent to the LCMV system is the class I-restricted, Lyt 2^+ effector arm of the T-cell system, since everyone agrees that these cells play a major effector role here (VARHO et al. 1981; BYRNE and OLDSTONE 1984; ALLAN and DOHERTY 1985; BAENZIEGER et al. 1986). With regard to this subset, it has been argued that their antiviral activity is mediated through the direct lysis of infected cells before infectious progeny are assembled, implicating cytotoxicity as the crucial function of this subset (ZINKERNAGEL and ALTHAGE 1977). This has been shown to work in vitro, and recent studies with cytotoxic influenza-specific clones, which show unique strain specificity in virus clearance in vivo (LUKACHER et al. 1984), appear to support this hypothesis. However, Lyt 2^+ Tc clones – including those LCMV-specific clones analyzed so far (BAENZIEGER et al. 1986) – often have a number of additional functions that might also seem relevant to virus clearance, such as the capacity to release gamma-interferon and MAF and to mediate DTH (KELSO et al. 1982; MORRIS et al. 1982; PRYSTOWSKY et al. 1982; MACDONALD et al. 1983; VON BOEHMER et al. 1984). Furthermore, when two virus-specific Tc clones were tested for virus-clearing ability in vivo, only the clone that also mediated DTH and produced gamma-interferon was effective (TAYLOR and ASKONAS 1983), suggesting that cytolytic capacity is not the only function required by this subset to mediate virus clearance. This conclusion is also supported by the finding that Tc cells fail to reduce virus titres in cyclosporin A-treated recipients (SCHILTKNECHT and ADA 1985).

The classical studies by BLANDEN (1971) on murine ectromelia virus infection suggest that attraction and activation of monocytes may be crucial, since adoptive transfer of effector T cells was markedly poorer in clearing the infection of recipients that had been preirradiated with 800 R. In an earlier study by our group, similar results were obtained in the LCMV system when the effect on liver titers was examined (THOMSEN and VOLKERT 1983). However, to prove the involvement of monocytes, lead-shielding of the bone marrow should protect the mice against the negative effects of preirradiation, but in a pilot experiment this was not found to be the case in our system (unpublished result). Although this may be attributed to the marked "antihemopoietic" effect of LCMV (BRO-JØRGENSEN 1978), it does raise the question whether preirradiation acts in some

other way that is irrelevant to the actual process of virus elimination. Here, the evidence supporting disruption of trafficking in irradiated mice is convincing (ANDERSON and WARNER 1976). It should be mentioned that BYRNE and OLDSTONE (1984) have found that LCMV-specific Tc clones were capable of causing virus clearance in the spleens of irradiated mice. This finding would seem to argue against the involvement of moncytes but, since the target organ was the spleen, it cannot be excluded that the macrophages already present might have been sufficient (TRIPATHY and MACKANESS 1970).

The suggestion that virus clearance in the LCMV system reflects a DTH-like reaction rests mainly on the results obtained in two models recently employed in our laboratory. In one system mice were pretreated with cyclophosphamide (Cy) 2 days before i.p. virus inoculation (THOMSEN et al. 1983b). With a dose of 150 mg/kg body weight, it was found that virus clearance was delayed in spite of the fact that splenic Tc activity was generated at a level which was comparable if not slightly higher than in untreated mice (Table 3). When the ability to raise a DTH response was tested, it could be shown that Cy-pretreated mice were deficient in this respect (Table 3), although spleen cells from these animals did induce a virus-specific DTH reaction when transferred to naive recipients challenged in the footpad (THOMSEN et al. 1983a). This finding, together with a reduced ability of Cy-pretreated, infected mice to support a DTH reaction to an unrelated antigen when this antigen and specific Td cells were inoculated locally, suggested that ancillary cells like monocytes were in short supply. Earlier BLANDEN and MIMS (1973) found that the LCMV-specific immune response is accompanied by macrophage activation. Therefore, when pre-

Table 3. The effect of cyclophosphamide (Cy) pretreatment on virus clearance, LCMV-specific cytotoxicity, and DTH[a] (modified with permission from the *Scandinavian Journal of Immunology* (1983) 17:489–495)

Mice	Cytotoxicity against L cells[b]		Increase in footpad thickness[c]			Blood virus titer[d]		
	LCMV-infected	Uninfected	24 h[e]	48 h	72 h	d6[f]	d9	d12
Untreated	147	0	58	65	31	1.3	1.0	−0.5
Cy-pretreated	166	0	0[g]	24[g]	17	1.8	2.5[g]	1.4[g]

[a] Groups of mice were given Cy (150 mg/kg) or left untreated. Two days later all mice were infected i.p. with 10^3 LD_{50} LCMV. At intervals blood from animals of both groups was assayed for virus titer. Eight days after infection, splenic LCMV-specific cytotoxicity was determined, and the DTH response was evaluated through direct challenge of mice in their left hind footpad with 10^6 LCMV-infected L cells

[b] LU_{50} per spleen

[c] Median of groups of four mice; mm $\times 10^{-2}$. Background (footpad swelling in uninfected controls) has been subtracted

[d] Log_{10} LD_{50}/0.03 ml blood

[e] Time after challenge

[f] Time after virus inoculation

[g] $P<0.05$; Mann-Whitney rank test, Cy-pretreated vs untreated mice

Table 4. Effect of macrophage blockade upon viremia (reprinted with permission from the *Scandinavian, Journal of Immunology* (1985) 17:489–495)

Treatment[a]	Blood virus titer[b]		
	Day p.i.	Test group	Controls
Carrageenan 0.5 mg i.p.	9	4.1(3.0–5.0)[c]	1.4 (NT–2.7)
Carrageenan 0.5 mg i.p.	10	4.0(4.0–4.4)[c]	NT (NT–NT)
Silica 5 mg i.v.	9	3.7(NT–4.4)[c,d]	1.0 (NT–2.3)[d]

[a] Mice in test groups received either 0.5 mg of carrageenan i.p. or 5 mg of silica i.v. 2 h prior to virus inoculation. Controls received virus only. Blood virus titer was determined on day 9 or 10 postinfection

[b] Median (and range) of minimum 4 mice; $\log_{10}$ plaque forming units/ml; *NT*, no trace

[c] $P<0.05$; Mann-Whitney rank test vs untreated controls

[d] Pooled results from two experiments

treatment with silica and carrageenan, both of which are known inhibitors of macrophages, also resulted in prolonged viremia (Table 4), apparently without suppressing the Tc (or antibody; unpublished results) response, we advanced the hypothesis that mononuclear phagocytes were critically involved in the clearance of LCMV (THOMSEN et al. 1983b). Yet further studies revealed that carrageenan was effective only when given early enough to affect the initial virus spread and had little effect when given at the beginning of the T-cell dependent phase or to naive recipients transplanted with primary effector T cells (THOMSEN and VOLKERT 1983). In view of this, it seems relevant to consider other conceivable explanations for the retarded virus clearance noted in Cy-pretreated mice.

If antibodies play an ancillary role in clearance of acute LCMV infection – and there are no data to refute this possibility – an effect on antibody production would be a possibility, since Cy is a potent inhibitor of B-cell responses (TURK and PARKER 1982). Also a direct effect on a critical T-cell subset remains a possibility; ALLAN and DOHERTY (1985) showed that primary effector cells from Cy-pretreated LCMV-primed donors, despite normal cytotoxic acitivity, were significantly inferior to cells from untreated donors in the capacity to clear virus from the brains of immunosuppressed recipients. In fact, the possibility exists that the primary defect in Cy-pretreated animals is an impaired capacity to clear the virus, and that the peripheral DTH unresponsiveness (anergy) results from the trapping of T cells and monocytes in the viscera.

In the second model, mice were inoculated with a relatively high dose of virus, viz 10^4 LD_{50} i.v. (or i.c.). Compared with mice infected with only 10^2 LD_{50} LCMV, high-dose mice showed markedly delayed virus clearance. Even 4 weeks after virus inoculation, spleen titers in most high-dose mice exceeded a level not found in low-dose mice later than 2 weeks after virus inoculation (MARKER et al. 1985; unpublished results). Perhaps more striking is the fact that virtually no drop in spleen titers is seen between day 5 and day 11 (Table 5), despite the induction of a potent Tc response only slightly inferior to that measured in low-dose mice (approx. twofold). Similar results were obtained with regard to brain titers in i.c. infected high-dose mice (THOMSEN and

Table 5. Virus clearance correlates with DTH reactivity not cytotoxicity[a] (reprinted with permission from the *Scandinavian Journal of Immunology* (1986) 24:137–145)

Virus dose	Specific ^{51}Cr release (%)			Increase in footpad thickness[b]			Spleen titer[c]		
	40:1	20:1	10:1	24 h[d]	48 h	72 h	d5[e]	d9	d11
10^2 LD_{50}	107	94	69	83	67	22	5.1	3.4	2.0
10^4 LD_{50}	93	68	43	3[f]	−1[f]	−16[f]	4.9	4.2	4.0[f]

[a] Groups of mice were infected with 10^2 or 10^4 LD_{50} of LCMV. At intervals spleens from mice of both groups were assayed for virus content. Eight days after infection, splenic cytotoxicity against LCMV-infected L cells was determined and DTH reactivity was evaluated through direct challenge of mice in their left hind footpad with 10^6 LCMV-infected L cells

[b] Median of groups of 5 mice, mm $\times 10^{-2}$. Background (footpad swelling in uninfected controls) has been subtracted

[c] Log_{10} LD_{50}/0.03 ml of 1:10 organ suspension

[d] Time after challenge

[e] Time after virus inoculation

[f] $P<0.05$; Mann-Whitney rank test, high-dose vs low-dose mice

MARKER 1986). The delayed virus clearance in high-dose mice does not seem to reflect a delayed antibody response, nor does it seem to reflect an increased virus load, since the spleen titer is not markedly higher in high- than in low-dose mice 5–7 days after virus inoculation, i.e., at the time when the immune effector functions normally set in (MARKER and VOLKERT 1973). When the LCMV-specific DTH response was tested, little or no reaction was found initially; slowly these mice gained the ability to raise a DTH response, and at the same time virus titers declined (MARKER and THOMSEN 1986). The correlation between DTH and virus clearance was further underscored by the observation that transfer of low-dose memory cells normalized the capacity both to raise a DTH response and to clear virus (THOMSEN and MARKER 1986). The latter observation also argues against the possibility that the virus in high-dose mice should be present in a changed or concealed form, and thus not in reach of the immune response. To ensure that the inability of high-dose mice to produce an LCMV-specific DTH reaction was not merely a peripheral deficiency of Td cells due to trapping, splenocytes from high-dose animals were transferred to naive recipients challenged at the same time in the footpad. No DTH reaction developed during the first 72 h, thus, indicating a selective deficiency of Td cells in high-dose mice. However, the transfer into naive recipients allowed high-dose cells to mature into effector Td cells through an X-ray sensitive step (probably reflecting proliferation), and at 96–140 h a DTH response emerged that was not seen in recipients given unprimed cells; at the same time these cells developed antiviral capacity in their new hosts (THOMSEN and MARKER 1986).

One additional finding that ought to be mentioned is the observation by ALLAN and DOHERTY (1985) that primary effector cells treated with anti-Lyt 2 and complement – and thus depleted of Tc cells – although capable of mediating meningeal inflammation, cannot accomplish virus clearance in the brain. Although it might be argued that this indicates the irrelevancy of Td function

to the process of virus clearance, we interpret this finding as a reminder that it is not the inflammatory reaction per se which brings about the reduction in virus titer. Probably direct cytotoxicity is mandatory, but in addition local production of lymphokines is crucial, and it is the latter requirement that constitutes the basis for the observed correlation with DTH.

LEHMANN-GRUBE and coworkers (1985) have recently opposed the idea of DTH being relevant to the clearance of LCMV. They base their argument on the observation that in their transfer model it is possible to obtain a reduction of spleen virus titer within 6–8 h. Clearly, within such a short period, there would not be sufficient time to develop a classical DTH reaction. However, in the spleen, a sufficient number of ancillary cells may already be present, and it is believed that the recruitment of these cells constitutes the basis for the time lag in the development of a DTH reaction (MACKANESS 1971). These authors (LEHMANN-GRUBE et al. 1985) also stated that no activation of macrophages can be detected in the adoptively immunized recipients. However, according to the design of their assay, very few cells are infected at the time of cell transfer, and this may explain why so few T cells in a very short time are capable of controlling the infection. Therefore, one might argue that so few macrophages are involved that no alterations would be detectable whichever mechanism might be at work. Furthermore, it should be kept in mind that virus clearance in the intact animal might be a more complex process than realized from such adoptive transfer systems (NASH and ASHFORD 1982).

3.2.1 Survival and DTH Reactivity in the Immune Modulated Host

Clinical recovery from viral infections generally results from the overall effect of the host's immune system, but occasionally it is delayed, impeded, or even prevented by immune-mediated lesions induced by the infecting agent. Presumably, such dualism of an antiviral T-cell response is more clearly exemplified in murine LCMV infection than in any other virus model system.

Thus, COLE et al. found that virus-specific sensitized spleen cells can protect mice subsequently challenged with i.c. inoculated LCMV. However, they could also show that similar cells reverse the Cy-induced protection of i.c. infected mice by inducing fatal LCM disease in such animals, and that this effect can be abrogated by pretreatment of the transferred cells with anti-theta serum (COLE and NATHANSON 1974; NATHANSON et al. 1975).

We also have evidenced that specifically sensitized T-lympocytes not only confer LCM disease, but also protect against this condition (VOLKERT et al. 1974; MARKER et al. 1976; THOMSEN et al. 1979). Furthermore, these results strongly suggest that the kinetics of the T-cell response related to that of virus multiplication are decisive for the final outcome.

The demonstration of virus-specific Tc cells in vitro (MARKER and VOLKERT 1973; ZINKERNAGEL and DOHERTY 1973) afforded grounds for the idea that the in vivo effect of such cells is a decisive mechanism in the elimination of an LCMV infection and an number of other infections. This question is discussed in detail above (Sect. 3.2). Moreover, it was also suggested – and generally accepted – that a key event in the development of fatal LCM disease could

be a direct interaction between such Tc cells and vital virus-infected targets in the central nervous system (CNS). This would take place mainly in heavily infected structures in arachnoidea and the choroid plexus epithelium and subsequently lead to a disadvantageous composition of the cerebrospinal fluid, which under nonpathological conditions is strictly controlled (DOHERTY and ZINKERNAGEL 1974; MARKER et al. 1976).

Several findings substantiate this view:

1. Virus-specific Tc-cell activity parallels the development of the condition (MARKER and VOLKERT 1973; ZINKERNAGEL and DOHERTY 1973)
2. Tc cells can be demonstrated in the cerebrospinal fluid of diseased animals (ZINKERNAGEL and DOHERTY 1973)
3. Increasing virus-specific cytotoxic activity in splenocyte populations correlates with increasing ease in demonstrating their ability to induce fatal meningitis in immunosuppressed mice (DOHERTY and ZINKERNAGEL 1975)
4. The activity of these cells both in vivo and in vitro is dependent on class I histocompatibility with the target (DOHERTY et al. 1976)
5. Long-term cloned LCMV-specific Tc cells have been shown to mediate LCM disease and death shortly after inoculation of the cells directly into the brains of i.c. infected, immunosuppressed recipients (BAENZIEGER et al. 1986)

In spite of this strong evidence, several experimental situations have recently been described, in which there is an obvious lack of correlation between Tc activity and in vivo lethal potential. PFAU et al., using an aggressive strain of LCMV that kills following i.c. inoculation and a docile one that does not, found that virus-specific cytotoxic activity in the meningeal exudate of mice infected with the docile virus ranged from 50%–100% of that found in the exudate of mice inoculated with the aggressive strain. They also showed that the exudate cells from mice infected with the aggressive strain caused lethal disease when transferred to irradiated recipients infected with either strain. In contrast, this effect could not be demonstrated with cells from the meningeal exudate of mice infected with the docile LCMV strain (PFAU et al. 1985). This group also noted that DTH appeared to be inhibited in docile-strain infected mice, intraplantar inoculation of the docile strain did not result in any footpad swelling on day 8, as compared with a twofold increase in similarly challenged mice infected with the aggressive strain. By use of in vitro restimulated memory cells, they indentified the disease-mediating T cells as Lyt 1^+2^+. This phenotype was the same as that of *K, D* region-restricted memory cells mediating DTH in irradiated mice. Furthermore, activated macrophages, which are essential for the manifestation of DTH, were found capable of enhancing the DTH response and accelerating symptoms induced by adoptive transfer of memory cells (PEVEAR 1984).

Using Cy-immune modulation we have previously tried to establish a role for DTH in fatal LCMV-induced meningitis. Pretreatment with 150 mg/kg protected 80%–90% of mice infected i.c. against acute LCM disease (THOMSEN et al. 1983a).

Analysis of the effect of Cy pretreatment on the virus-specific immune response revealed that Tc activity was apparently unaffected, whereas DTH reac-

tivity was markedly depressed, and that this reflects a lack of ancillary cells required for the expression of DTH. However, most of the latter experiments were carried out using i.p. infected animals, and it could be questioned whether the results obtained in this way would be valid for i.c. inoculation. Thus, when more experiments were performed with i.c. infected mice, it was found that in these animals Cy did suppress the Tc response slightly; the Tc activity peaked at a level that was about 50% of that in untreated animals (unpublished results). A twofold difference is, however, not likely to explain the marked difference in mortality. More importantly, when meningeal infiltration was evaluated in Cy-pretreated animals, we did not find a marked difference between these mice and untreated i.c. infected mice. Consequently, we cannot maintain the original hypothesis that Cy pretreatment protects against LCMV disease simply through depletion of ancillary cells (THOMSEN et al. 1983a), and the mechanism underlying the reduced mortality is uncertain at present. One observation may be relevant in this context: DOHERTY and ALLAN found that splenocytes from Cy-pretreated, LCMV-primed donors protected mice infected i.c. with a low dose of virus as effectively as did donor cells from untreated mice. However, whereas the latter cells did so through an early abortion of the infection, mice given cells from Cy-pretreated donors survived despite virus persistence in the brain (DOHERTY and ALLAN 1985). Therefore, in the latter case the transferred cells must have suppressed the otherwise lethal antiviral response.

In the high-dose LCMV model (Sect. 3.1), 90% of mice given the high-dose survived the infection, whereas mice similarly inoculated with a low dose invariably died from fatal LCM (Table 2). It was demonstrated that virus-specific T-cell cytotoxicity was expressed to an equal degree in mice given high or low doses irrespective of (a) route of infection and (b) origin of effector-cell suspensions, i.e., from the spleen, lymph nodes, peripheral blood, or the meningeal exudate. However, direct challenge of mice receiving high doses of virus revealed no appreciable DTH reactivity (MARKER et al. 1985), and adoptive transfer established that these mice had few or no effector T cells. These results implied a suppression of Td function in animals given the high dose as one immunological explanation for their survival. Consequently, a fully developed Td function could be suggested as an alternative precondition for the development of fatal LCM.

Another possible explanation of survival after a high dose might be that LCMV antigen was presented differently on the surfaces of virus-infected cells in the brains of high- and low-dose mice. On the other hand, immunocytochemical studies of the choroid plexus from high- and low-dose mice revealed a similar intensity and distribution of the virus infection in the two groups of animals (MARKER et al. 1985). Moreover, when spleen cells obtained from low-dose mice either (a) at the peak of the virus-specific cytotoxicity (MARKER and VOLKERT 1973) and primary DTH reactivity (TOSOLINI and MIMS 1971) or (b) 2 or 3 months later were transferred to i.v. infected high-dose animals, they restored the capability of these animals to produce a strong footpad swelling in response to locally injected LCMV (Table 6). When similar spleen cells were transferred to i.c. infected high-dose recipients, classical fatal LCM disease developed without exception (MARKER and THOMSEN 1986). Thus, it seems beyound

Table 6. The ability of sensitized spleen cells (from low-dose mice) to mediate DTH and LCM disease in high-dose mice (reprinted with permission from the *Scandinavian Journal of Immunology* (1986) 24:127–135)

Dose of virus infection of recipients[a]	Cells transferred i.v.	Increase in footpad thickness[e]			Mortality (dead/total)
		24 h	48 h	72 h	
10^4 LD_{50}	Nil	26	24	9	1/5
10^4 LD_{50}	100×10^6 Primary precursors[b]	27	20	15	1/5
10^4 LD_{50}	100×10^6 Primary effectors[c]	91	95	39	5/5
10^4 LD_{50}	50×10^6 Memory cells[d]	78	42	11	5/5
10^2 LD_{50}	Nil	101	104	39	5/5

[a] Recipients were infected i.v. (for the DTH assay) and i.c. (for the mortality experiments) 2 days before i.v. transfer of the cells

[b] Donors were infected i.v. with 10^2 LD_{50}LCMV, 2 days prior to harvest and i.v. transfer of spleen cells

[c] Donors were infected 8 days prior to harvest and i.v. transfer of spleen cells

[d] Donors were infected 90 days prior to harvest and i.v. transfer of spleen cells

[e] Recipients were challenged with 1×10^6 LCMV-infected L cells in the right footpad 8 days after the systemic virus infection (6 days after cell transfer). Increase in footpad thickness was measured 24, 48, and 72 h after challenge and is expressed in mm $\times 10^{-2}$

doubt that the LCMV-infected CNS structures in i.c. inoculated high-dose mice are sensitive to the in vivo effect of committed spleen cells with fully developed Tc and Td function. Bearing in mind the abundance of Tc cells in meninges of high-dose mice, this finding strongly suggests, albeit indirectly, that the Td function is a critical type of immune response in LCM disease.

Since we have determined the primary T-effector cells responsible for the LCMV-specific DTH reaction to be of the Lyt 1^+2^+ phenotype (THOMSEN and MARKER 1986), the above findings are consistent with the view held by, among others, ALLAN and DOHERTY (1985) that fatal LCMV disease results from cell damage caused by Lyt 2^+ cells. These authors, on the other hand, find that the severity of the meningeal inflammation (as measured by number of cells/μl of cerebrospinal fluid) is not diminished by the removal of Lyt 2^+ subsets from LCMV-immune lymphocytes transferred to Cy-pretreated infected recipients (ALLAN and DOHERTY 1985). This is in accordance with our histological demonstration of an equal degree of cellular infiltration in brains from high- and low-dose mice (unpublished results). Consequently, the clinical course of the infection is not dependent on the mere abundance of cells in the cerebrospinal fluid or the virus-specific cytotoxic potential of such cells, but rather on the presence of T cells with certain additional crucial qualities. Indeed, the meningeal exudate of mice fatally infected with LCMV has been shown to contain T cells, which have the capability to mediate virus-specific DTH (SARON and GUILLON 1983). It is therefore of relevance that PFAU and coworkers have found marked differences between the meningeal exudate macrophages derived from fatally infected animals and those harvested from docile LCMV-infected mice (PFAU et al. 1985).

Somewhat parallel to this OLDSTONE and DIXON (1971b) years ago noted that depletion of C3 (with cobra venom factor, CoF) delayed death following i.c. infection. Since CoF treatment affects the nonspecific components of DTH (JUNGI and PEPYS 1981), both observations appear to support the hypothesis that fatal meningitis is a DTH-like reaction. How this may be reconciled with the finding that cloned Tc cells are capable of inducing fatal disease in recipients apparently unable to support a DTH reaction is difficult to say. However, two possibilities might be worth considering. First, the direct injection of large numbers of cloned T cells into the brain may bypass a need for an amplifying step crucial under more "natural" conditions. Alternatively, it could be speculated that components of the inflammatory exudate in non-fatally infected animals might have a protective capacity.

It should be mentioned that our idea of a DTH reaction being responsible for tissue damage in a virus infection is not unique. Lung lesions in mice infected with influenza virus are similarly attributed to the effect of virus-specific Td cells (LIEW 1984). Also evidence suggests that the demyelinating disease induced by THEILER murine encephalomyelitis virus reflects a virus-specific DTH reaction (CLATCH et al. 1985).

3.2.2 Neuropathological Studies of LCMV-Induced Choriomeningitis

Convulsive seizures immediately before death are characteristic of the final stage of LCMV disease, and these dramatic symptoms have prompted an interest in the relevant pathology.

Light microscopy reveals cellular infiltrations of varying degree, located mainly in the pia, arachnoidea, and choroid plexus. The infiltrate is dominated by mononuclear cells of varying size and constitutes the most prominent pathological finding (LILLIE and ARMSTRONG 1945). The obvious discrepancy between the rather discrete histopathological findings and the dramatic symptoms of the illness applies not only to LCMV infection but to many other virus infections of the CNS in humans (BOYD 1961) as well as experimental animals. Therefore, recent studies of the pathology of LCMV-induced choriomeningitis have been concentrated on attempts to visualize the results of the immunological interactions underlying the disease (CAMENGA et al. 1977; WALKER et al. 1977; SCHWENDEMANN et al. 1983). A search for direct evidence of the interaction between Tc cells and, in particular, epithelial cells of the choroid plexus (where the infection is most intense) has been the object of several studies (CAMENGA et al. 1977; WALKER et al. 1977; SCHWENDEMANN et al. 1983). WALKER and coworkers found no cytopathological changes in the epithelial cells of the choroid plexus of fatally infected but otherwise untreated mice. However, anticonvulsive treatment with diazepam prolonged the lives of these mice by several days, and in such treated mice, the authors observed cytopathological changes in the choroidal epithelial cells, ranging from dilated endoplasmic reticulum to necrosis (CAMENGA et al. 1977; WALKER et al. 1977). SCHWENDEMANN et al. found infrequent necrosis in the plexus epithelium of fatally LCMV-infected mice (SCHWENDEMANN et al. 1983). These authors also claim that areas of contact between lymphocytes and epithelial cells corroborate the theory of "lethal hit". However,

neither the intimacy of the contact nor the characterization of the presumed effector cells can justify such a bold conclusion. Similar intimate contacts are well described in other situations of interaction between immunologically active lymphocytes and macrophages (NIELSEN et al. 1974).

Using radioiodinated bovine serum albumin, DOHERTY and ZINKERNAGEL obtained results that afforded grounds for the idea of cerebral edema in LCMV disease (DOHERTY and ZINKERNAGEL 1974). Furthermore, they reported the pen-

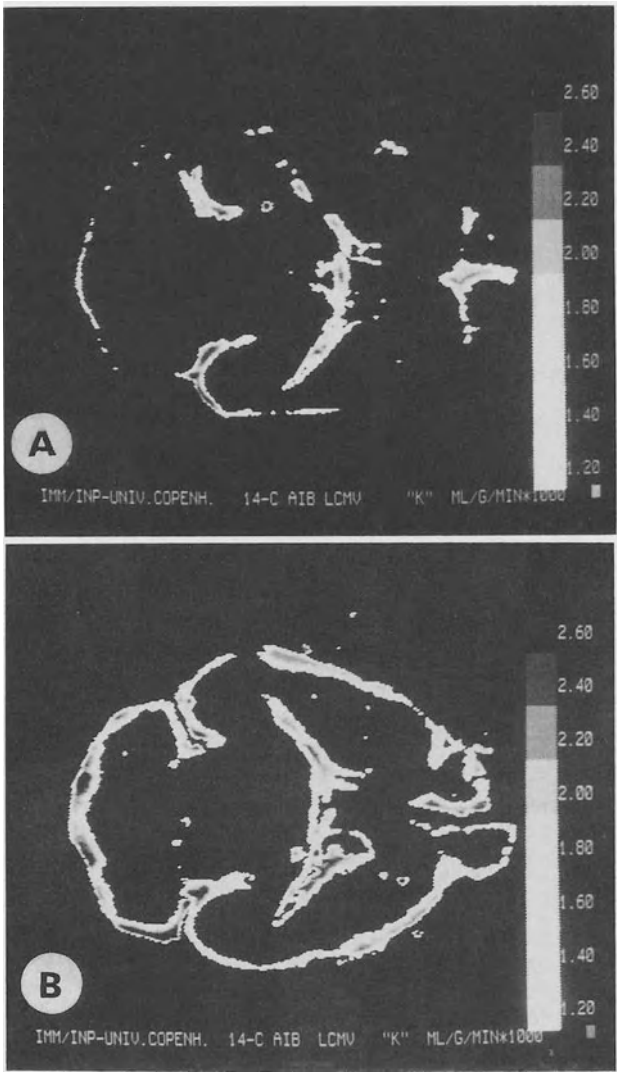

Fig. 1 A, B. [^{14}C]AIB autoradiograms of horizontal sections of mouse brains. **A** Brain of control mouse. **B** Brain of LCMV-inoculated mouse 7 days after infection. There is increased permeability to AIB in all leptomeningeal regions and around the choroid plexus. Scale units represent value of K_{in}, the transfer constant (ml/g/min × 1000) × 7.5. (reprinted with permission from *Acta Neuropathologica* (1984) 63:229–239

etration of Evans blue into the brain parenchyma. The conclusions drawn from these experiments concerning the brain edema have been questioned (CAMENGA et al. 1977), and neither we nor researchers from several other laboratories have been able to obtain results substantiating the theory of edema in the neuropil (CAMENGA et al. 1977; SCHWENDEMANN et al. 1983; MARKER et al. 1984). Interestingly enough, investigators in this field (G.A. COLE, personal communication; C.J. PFAU, personal communication) often remark on a loose, "edematous" consistency of brains from mice suffering from LCMV disease, although experimental evidence is sparse.

Using the tracers 2-amino (1-^{14}C) isobutyric acid and horseradish peroxidase (HRP) together with electron microscopy, we have demonstrated an obvious dysfunction of the blood-brain barrier (BBB) in LCM disease (MARKER et al. 1984). This dysfunction was seen in the choroid plexus and throughout the leptomeninges (Fig. 1), where a pronounced perivascular leakage of HRP was the most striking finding (Fig. 2)

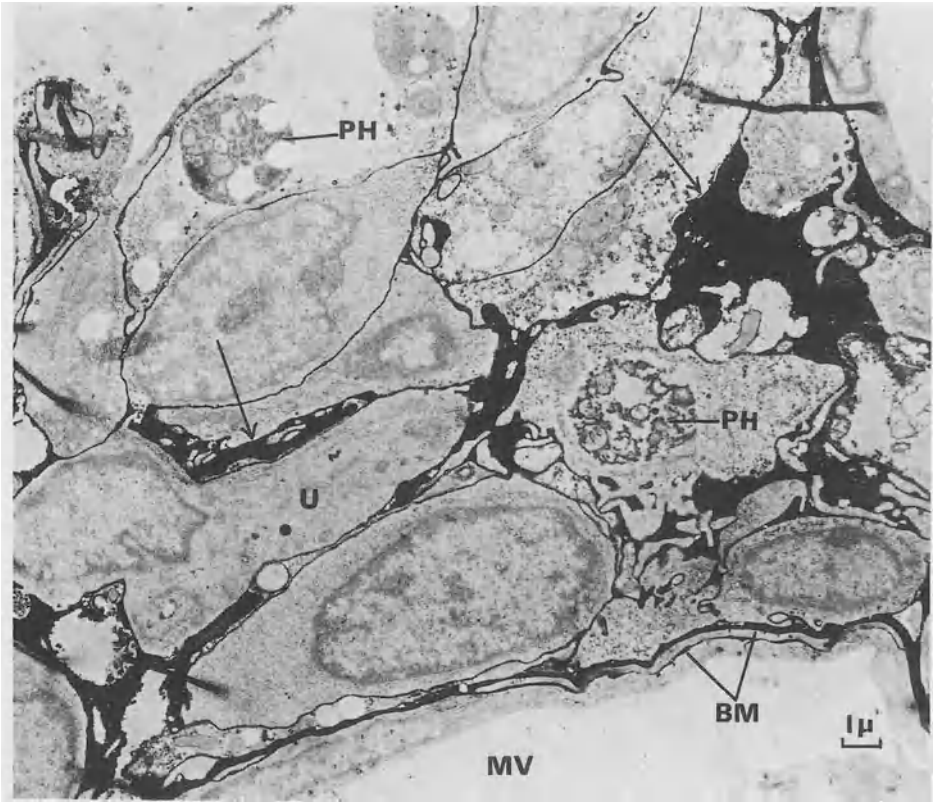

Fig. 2. Part of meningeal vessel (*MV*) from infected mouse. The cells accumulated in the subarachnoidal space are: activated lymphocytes, one of which displays an uropod (*U*), and macrophages with phagocytosed necrotic cells (*PH*). The electron-dense reaction product occurs in the space occupied by the basement membrane (*BM*), and in the extracellular fluid (*arrows*). (reprinted with permission from *Acta Neuropathologica* (1984) 63:229–239)

This increased vascular permeability distributed generally in the meninges is not likely to be the result of a local effect of cytotoxic cells. It seems much more likely that a soluble factor released from committed T-lymphocytes or from the accumulated monocytes could be responsible. Moreover, in unpublished experiments with HRP we have found that the plexus epithelial cells of fatally infected mice were almost uniformly strongly stained with this tracer, whereas in normal controls similar cells were only faintly stained. Also this finding is most easily explained by assuming the release of a factor that increases permeability.

Apart from increased vascular permeability with pronounced extravasation of fluid, we demonstrated: (a) infiltration with predominantly mononuclear cells, (b) many intravascular lymphocytes adhering to the endothelium, and (c) some of these lymphocytes penetrating the vessel wall (Fig. 3). Taken together, these changes are also those classically considered characteristic of a DTH reaction (David 1968). However, one should be wary of drawing conclusions with regard to the role of DTH in LCMV disease based on pathological anatomy alone. As stated in the above, involvement of direct cytotoxicity cannot be

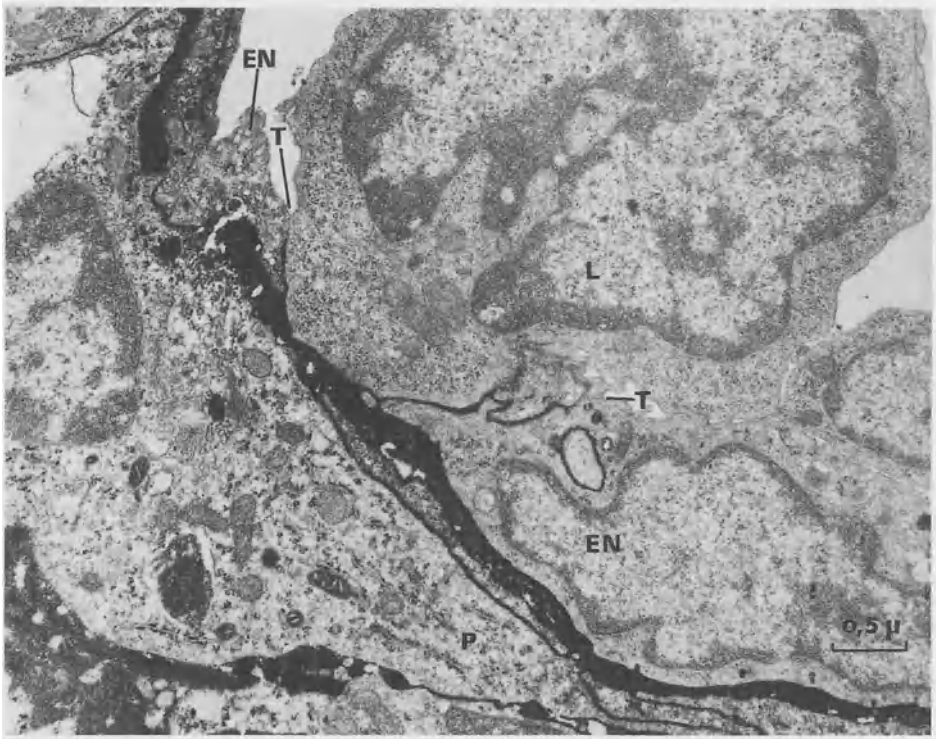

Fig. 3. A lymphocyte (*L*) from an infected mouse is seen to penetrate the endothelial cell (*EN*) of a meningeal vessel. A tight junction (*T*) is formed between the lymphocyte and the endothelial cell. Peroxidase reaction product is present in the space occupied by the basement membrane surrounding the proliferating pericyte (*P*) × 27800 (reprinted with the permission from *Acta Neuropathologica* (1984) 63:229–239)

ruled out on morphological grounds. The idea is strongly supported by the fact that Tc cells are present in the exudate (ZINKERNAGEL and DOHERTY 1973; MARKER et al. 1985), and also by results obtained with cloned LCMV-specific Tc cells causing death in virus-infected immunosuppressed mice (BAENZIGER et al. 1986). In addition, some of the above changes can, in various combinations, be found in any inflammatory reaction. On the other hand, a pronounced general breakdown of the BBB in the meninges and the choroid plexus, most likely to be induced by soluble factors, is a significant part of the pathological changes during LCMV disease and might constitute an additional element in the development of the fatal illness. Finally, the pathology of LCMV-induced choriomeningitis is compatible with a DTH reaction.

4 Specific DTH Unresponsiveness in Experimental Virus Infections

4.1 Virus-Specific Suppression of the DTH Response Mediated by Ts Cells

That a great number of viruses constantly cause infections and multiply in their favored host species could be considered a consequence of a certain balancing of their infective and pathogenic qualities. Since DTH constitutes a complex type of immune response which plays a role not only in the clearance of viruses but also in virus-induced, immune-mediated tissue damage (BLANDEN 1971; LEUNG and ADA 1982; CLATCH et al. 1985), it could be speculated that moderation of antiviral DTH reactivity its one of Nature's strategies for assuring the host's survival and, thereby, also the evolutionary success of the virus in question. Although this idea is hypothetical, the fact remains that "split tolerance," in which DTH is the suppressed arm of the immune response, is described for several viruses (ADA et al. 1981; NASH and GELL 1981; CLATCH et al. 1985; MARKER et al. 1985). Thus i.v. injections of HSV (NASH and GELL 1981) and LCMV (MARKER et al. 1985) may lead to situations in which virus-specific antibody production and Tc function are strongly expressed, but DTH reactivity is abolished. In many model systems, the effect of antigen-specific Ts cells has been demonstrated as an explanation of this unresponsiveness. This applies to sheep red blood cells (KAUFMANN et al. 1979), haptens (ASHERSON and ZEMBALA 1974), and several viruses (GREENE and WEINER 1980; LIEW and RUSSELL 1980; ADA et al. 1981; NASH et al. 1981a).

In murine herpes virus infection antigen-specific suppressor cells selectively directed towards the Td function can be demonstrated shortly after an i.v. infection and are shown to mediate suppression when transferred to normal recipients. In this system, the early suppressor cells are of the Lyt 1^+2^- phenotype, although later cells not only of this phenotype but also of the Lyt 1^-2^+ type are capable of conveying virus-specific suppression. Another striking feature of this infection is that i.v. inoculation leads to Td suppression, whereas s.c. inoculation is sensitizing in terms of a DTH response. The induced suppression seems in this case to be lifelong and is, furthermore, virus-type specific.

In murine influenza infection infectious virus given intranasally induces the development of T cells suppressing an *I* region-restricted DTH response without

interfering with antibody production (LIEW and RUSSELL 1980). Inactivated influenza virus, however, produces suppressor cells that inhibit the *K, D* region-restricted DTH response (LEUNG et al. 1980). An interesting feature of the rather complex murine influenza system is that DTH sensitization (*I* region-restricted) brought about by inactivated virus leads to immunopathological lesions in the lungs when the mice are subsequently challenged with live virus given intranasally (LEUNG and ADA 1980). This tissue damage could be prevented by the transfer to such animals of *I* region-restricted suppressor cells (LIEW 1983).

Similarly, in the Sendai virus model, infectious virus induces Ts cells that suppress the generation of *I* region (but not *K, D* region)-restricted effector cells. Inoculation of noninfectious F-Sendai virus, however, gives rise to a generation of Ts cells that suppresses a *K, D* region-restricted response on the Tc as well as the Td level, but does not influence an *I* region-restricted DTH response (ADA et al. 1981). The symmetry between the infectious state of the virus administered and the MHC restriction of the suppression induced, which appears in these models, is interesting with regard to the protective and pathogenic roles of the two types of T-effector cells expressed in spontaneous infection. Since LCMV is known to cause widespread infection of the lymphoid system, it may be speculated that the absence of an *I* region-restricted DTH response in this infection reflects an extreme case of a similar regulation directing T-cell induction towards a *K, D* region-restricted response (see also Sect. 2.2.).

By comparing the results of studying antigen-specific suppression in various systems, including DTH reactivity to haptens, GERMAIN and BENACERAF have integrated these findings into a general scheme of the events taking place when immune suppression is mediated by Ts (GERMAIN and BENACERRAF 1981). According to this synthesis, antigen activation of Ts precursors (Lyt 1^+ or 1, 2, 3^+) induces the maturation of Ts1, which is believed to be an afferent suppressor. A product of Ts1 is TsF1, which – together with antigen – makes Ts2 precursors develop into Ts2 (Lyt 2, 3^+). Ts2 cells then produce TsF2, which in turn stimulates Ts3 (Lyt $2^+ 3^+$) to produce TsF3, presumbably a nonspecific factor for efferent suppression of the manifestations of the immune response in question. This pathway many apply also to virus-induced, Ts-mediated suppresion of the DTH response (LIEW 1984).

4.2 DTH Tolerance Not Mediated by Ts Cells

Although a number of experimental models used in the study of antigen-induced tolerance have produced evidence for a central role of suppressor cells, this is not the case in all systems (ROITT et al. 1985). In tolerance induction a distinction must be made between irreversible and reversible unresponsiveness (SINCLAIR 1985). The classical explanation of irreversible tolerance is based on the theory of clonal deletion originally presented by BURNET (1959) and implies complete elimination of those cells of the immune system that carry specific receptors for the antigen in question. In reversible tolerance, on the other hand, responsiveness is reestablished in time, most probably as a consequence of a progressive antigen elimination and a resulting reappearance of the specific clones.

Not only suppressor cells but also noncellular entities have been found to regulate the immune response in experimental systems. Thus, serum antibody (VOISIN et al. 1969), anaphylactic antibody (VOISIN 1976), antigen-antibody complexes (WRIGHT et al. 1973), free antigen (BALDWIN and ROBINS 1976; LAMB et al. 1983), and various suppressor factors (GERSHON 1974) have all been connected with reversible or incomplete suppression of immune responses.

In spite of the fact that virus-induced immune tolerance has long been recognized as a characteristic feature of murine LCMV infection (HOTCHIN 1971; LEHMANN-GRUBE 1971), suppressor cells have so far not been demonstrated in this system (for example: CIHAK and LEHMANN-GRUBE 1978). The DTH tolerance induced in adult mice following infection with high doses of LCMV will be dealt with in some detail below.

When the duration of selective DTH unresponsiveness induced by high doses of LCMV was investigated, it was found to be maximal during the first 2 weeks; 4 weeks after virus inoculation, however, no statistically significant difference in the DTH responses of high- and low-dose mice could be demonstrated. At this time, virus elimination was complete in low-dose mice, and the virus content in spleens and kidneys of some, but not all, high-dose animals was considerably reduced (MARKER et al. 1985; MARKER and THOMSEN 1986). Thus, high-dose tolerance is seen to decline as virus clearance progresses.

That the tolerance is far from complete (i.e., clonal abortion) is also underlined by the finding that high-dose spleen cells harvested during DTH unresponsiveness, when transferred to normal recipients preinfected in the footpad, convey virus-specific DTH reactivity. The footpad response in this case, however, is not expressed during the normal 72-h observation period, but only when footpad thickness is measured over an additional period of 3 days. This finding is in accordance with the idea that primed Td precursor cells are indeed induced in high-dose mice, but that in this environment of high antigen concentration, their maturation into Td-effector cells, capable of mediating an LCMV-specific DTH reaction, is inhibited (MARKER and THOMSEN 1986; THOMSEN and MARKER 1986). Preirradiation of such high-dose cells revealed that this "late" DTH reactivity is dependent on cell proliferation following transfer to an animal harboring only small concentrations of virus (THOMSEN and MARKER 1986).

Since a low dose of virus given i.v. induces fully developed Tc and Td responses and is fatal when injected i.c., whereas a high dose spares the mice and selectively suppresses the Td response, i.c. inoculated low-dose animals seemed an obvious test system for possible virus-specific suppressor cells produced in high-dose mice. When high-dose spleen cells are transferred to i.c. infected low-dose mice, death of the recipients is in fact prevented, providing that donor cells are harvested at a time when spleen-virus titers peak and that recipients are in the early stage of the infection (Table 7). This seemingly afferent suppression is not MHC restricted, which implies that it is not caused by Ts cells (MALKOVSKY and MEDAWAR 1984), although the need for restriction might have been obscured by allogeneic effects or by the probably low degree of polymorphism of the *I-J*, *I-E/C* region alleles (MURPHY 1978). Another possibility would be that suppression is mediated by cells other than T cells, for example,

Table 7. Characterization of efferent suppression induced (in low-dose mice) by transfer of spleen cells from high-dose donors (reprinted with permission from the *Scandinavian Journal of Immunology* (1986) 24:127–135)

Donors			Pretreatment of cells transferred[c]	Recipients[b] mortality (dead/total)
Mouse strain	Route of infection	Dose of LCMV[a]		
C3H	–	Nil	Nil	5/5
C3H	i.c.	10^2 LD_{50}	Nil	4/5
C3H	i.c.	10^4 LD_{50}	Nil	0/5
BALB/c	–	Nil	Nil	5/5
BALB/c	i.c.	10^4 LD_{50}	Nil	0/5
C3H	i.c	10^4 LD_{50}	NMS[d]	2/10
C3H	i.c.	10^4 LD_{50}	Anti-LCMV[e]	10/10

[a] Spleen cell suspensions were obtained 4 days postinfection from all infected donors
[b] All recipients received spleen cells by the i.v. route 2 days after i.c. inoculation of 10^2 LD_{50} LCMV
[c] In all cases 100×10^6 spleen cells were transferred
[d] Normal mouse serum
[e] Serum from C3H mice containing neutralizing antibodies against LCMV

macrophages (Colizzi et al. 1984), NK cells (Katz 1984), or B cells (Gilbert and Hoffmann 1983). Therefore, it is pertinent that high-dose spleen cells lose their life-saving effect in fatally infected low-dose mice when they are pretreated with neutralizing mouse anti-LCMV serum (Table 7). Following this lead, a reduction of mortality was attempted by i.v. inoculation of high doses of stock virus. Depending on the time interval between primary i.c. virus inoculation and an i.v. superinfection, death could be prevented to varying degrees. When 10^7 LD_{50} LCMV is given on day 4 postinfection, 90% of otherwise fatally infected mice survive (Fig. 4). In this context it is also of interest to note the efficiency with which low-dose spleen cells produce fatal disease in i.c. infected high-dose mice (Sect. 3.3.1). In our view, this is not easily conceivable if efficient efferent suppression in high-dose mice is caused by a tolerance-inducing population of Ts cells.

Our interpretation of the above results is that the selective DTH tolerance induced with high doses of LCMV is due to modulation of the immune response induced by an interaction between antigen and antigen-sensitive T cells without the involvement of Ts cells. This interaction results in priming of virus-specific Td precursors, but prevents their clonal expansion.

Another possibility would be that LCMV-specific Ts cells are induced during infection, but are so few or so weakly expressed that they escape notice in adoptive transfer systems. If suppression is looked upon in terms of network regulation, this is not altogether unlikely. Cooper and Eichmann (1985) have presented data indicating that T-cell unresponsiveness to trinitrophenyl (induced by i.v. injection of trinitrobenzene sulfonate) reflects a state of increased connectivity – and hence suppression – in the network that normally keeps the immune system in a steady state. If this applies also to high-dose LCMV infection, it is not surprising that the dispersion of the cells and their transfer to a new

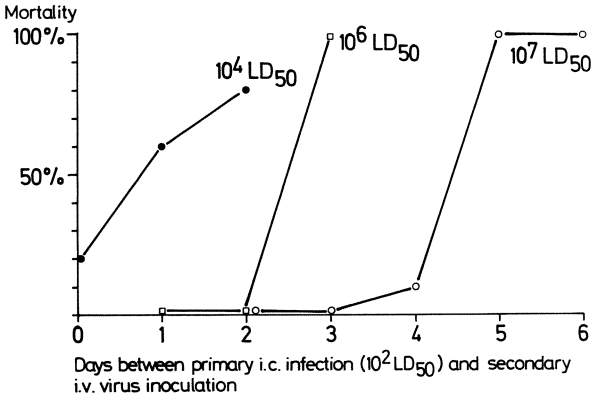

Fig. 4. The mortality (%) in groups of mice infected i.c. with 10^2 LD_{50} of LCMV on day 0, and later unjected i.v. with 10^4 LD_{50} of the same stock virus on days 0, 1, or 2 (●——●); 10^5 LD_{50} on days 1, 2, or 3 (□——□); or 10^7 LD_{50} on days 2, 3, 4, 5, or 6 (○——○) (reprinted with permission from the *Scandinavian Journal of Immunology* (1986) 24:127–135)

environment, which presents the viral antigen(s) optimally, reverses the unresponsiveness.

The mechanism, by which a reversible, functional "clonal anergy" (NOSSAL and ADA 1971) is established, is as yet obscure and a matter of speculation. On the other hand at least three different possibilities often mentioned in this context may be worth considering: first, it is widely accepted that optimal T-cell responses are elicited when antigen is presented by macrophages, dendritic cells, or other types of Ia^+ antigen presenting cells. In situations where the amount of free antigen exceeds the capacity of the reticuloendothelial system, this free antigen may gain direct access to T cells carrying specific receptors on their surfaces. Such interaction might result in a signal, that induces unresponsiveness rather than response. Second, antigen-antibody complexes are considered effective inducers of immunological tolerance (BANSAL et al. 1973a, b). Third, various, vaguely defined suppressive factors have been claimed to be of importance.

In high-dose mice, virus replication and interferon production are enhanced as compared with that in low-dose animals (MARKER et al. 1985). In addition, we have been able to demonstrate that antibody production in high-dose mice is in fact accelerated, seemingly as a consequence of an early switch from IgM to IgG (unpublished results). Accordingly, it is conceivable that large amounts of free antigen, early appearing antigen-antibody complexes, as well as undefined nonspecific suppressive factors produced in the heavily infected high-dose mice may be cooperative in the induction of unresponsiveness (TAYLOR 1984).

If the idea of immune suppression in high-dose LCMV infection without the development of Ts cells is accepted, it could be argued that in nature there is no urgent need for a strongly expressed T-cell mediated suppressor mechanism. In permanently infected mouse colonies, namely, the offspring are infected by – among other excretions – urine from their mothers, infection takes place presumably with moderate doses of virus that replicate rapidly to high titers

in many organs, including spleen, lymph nodes, and thymus. In this way the stage is set for high-dose tolerance. The result of this process is the generation of persistently infected mice, whose infection affects them only slightly and, more importantly, does not interfere markedly with their reproduction. In other words the symbiosis between host and parasite is kept in perfect homeostasis by means other than active suppression of a fully developed immune system.

5 How many T-Effector Subsets Are Induced During Acute LCMV Infection?

The relative importance of Tc and Td functions in recovery from LCMV infection has been discussed in detail in Sects. 3.2 and 3.3.1. The central role generally attributed to virus-specific Tc cells is based mainly on:

1. The correlation between Tc function and virus clearance (MARKER and VOLKERT 1973)
2. Sparse experimental evidence of Tc cells destroying infected target cells before the release of infectious virus (ZINKERNAGEL and ALTHAGE 1977)
3. The virus-eliminating effect in various systems, including LCMV (BYRNE and OLDSTONE 1984), of cloned virus-specific Tc cells

The importance of Td cells, on the other hand, is mainly based on:

1. The rapid clearance of herpes virus following the transfer of Lyt 1^+2^- cells mediating DTH (NASH and GELL 1983)
2. The reduced clearance of LCMV when the DTH reactivity is selectively suppressed either by Cy (THOMSEN et al. 1983b) or by high doses of virus (MARKER et al. 1985; THOMSEN and MARKER 1986)

In an attempt to summarize our result on Tc and Td function in relation to virus elimination, we have collocated the time course of these variables in Fig. 5. This collocation emphasizes that maximal virus reduction is taking place when Tc and Td functions are both strongly expressed during the 2nd week after inoculation of relatively low, sensitizing doses of virus and may thereby suggest that a discussion of the separate importance of the two types of responses is somewhat academic. Additionally, the time course of the appearance of Tc and Td cells does not allow a distinction between these T-cell functions, provided optimally sensitizing doses of virus are used. This observation may raise the question whether they indeed constitute two distinct subsets of T cells or only one bifunctional subset. The idea of an antigen-specific bifunctional subset is supported by several findings. First, LCMV-specific Tc as well as Td function is *K, D* region-restricted (DOHERTY et al. 1976), and both functions are mediated by Lyt 2^+ T cells (VARHO et al. 1981; THOMSEN and MARKER 1986). Second, multifunctional T-cell clones have been demonstrated in several laboratories (BIANCHI et al. 1981; DENNERT et al. 1981; KAUFMANN and HAHN 1982). In several systems, including virus infections, clones have been isolated expressing

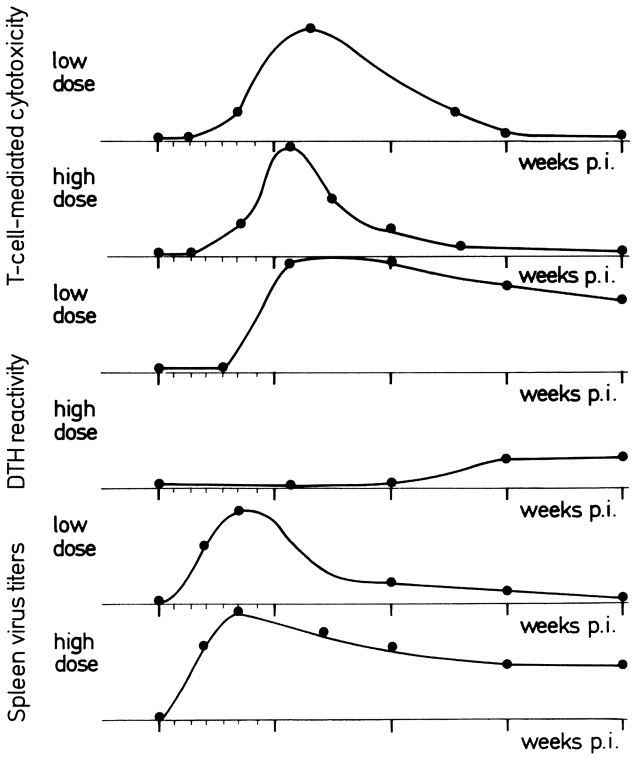

Fig. 5. The time course of critical parameters in mice infected with high (10^4 LD_{50}) or low (10^2 LD_{50}) doses of LCMV. *p.i.*, postinfection

cytotoxic activity as well as DTH reactivity (LIN and ASKONAS 1981; VON BOEHMER et al. 1984; BAENZIGER et al. 1986). Furthermore, in the same systems cloned T cells have been demonstrated to change their type of functional expression during long-term culture (TAYLOR and ASKONAS 1983; VON BOEHMER et al. 1983, 1984).

In light of results obtained from our study of the LCMV high-dose model, a single bifunctional T-cell subset would be conceivable, assuming that Tc and Td functions in such a subset can be switched on and off independently (Fig. 6). In response to low doses of virus, effector cells expressing Tc as well as Td functions would appear early. One to three weeks later the cells have acquired memory quantity on the Tc level, while some of them still maintain effector function as to Td activity. Why Tc function is turned off at this stage is not clear; virus elimination per se is not a sufficient explanation, since a similar decline in Tc activity is found in the "persistently" infected high-dose mice. Later still, they are all memory cells with regard to cytotoxicity as well as to DTH response (Figs. 5 and 6). When the higher dose of virus is used, the T-effector cells are suppressed for a long time to remain on the precursor level with regard to DTH, while this dose is still permissive as to their development

Fig. 6. Suggested functional development of the anti-viral T-cell response during LCMV infection. The *second step* is only hypothetical in low-dose mice, while there is experimental evidence for its existence in high-dose mice. The *third step* seems suppressed in high-dose mice. Although the depicted development is deduced from the results of "bulk" assays (Fig. 5), and hence reflects the overall T-cell response, similar changes may occur within multifunctional T-cell lineages depending on the signals received. *C*, cytotoxic; *D*, DTH-mediating; *P*, precursor; *E*, effector; *M*, memory

into cytotoxic effectors. Later, when the virus load is considerably decreased, the situation becomes more akin to that in low-dose mice.

On the other hand, the more conventional view implying the existence of two distinct virus-specific effector T-cell subsets, one expressing Tc function and the other Td function (LIEW 1984), is of course still a plausible model in spite of the similarity in *MHC* region restriction and Lyt phenotype, which applies to the LCMV system.

Whichever model is correct, our results imply that activation of Tc and Td functions have distinct triggering requirements either in terms of antigen presentation or of nonspecific signals enhancing or suppressing proliferation and differentiation. The difference, however, seems to be of a quantitative rather than a qualitative nature. That is, if very high doses of virus are introduced, Tc function is turned off. Under these conditions Tc priming does occur, even though there is no Tc response in situ. Also in this case there is no transferable cell-mediated suppression (LEHMANN-GRUBE et al. 1982). These findings parallel completely our results with regard to Td function following inoculation of only moderately high doses of virus.

6 Concluding Remarks

By employing an immune-modulated host, predominantly produced by inoculation of moderately high doses of infectious LCMV, we have obtained experimental evidence supporting the view that DTH reactivity plays an important role in elimination of the virus and in LCMV-induced pathogenicity.

Basically, we have demonstrated optimal virus clearance and maximal mortality, depending on the route of virus inoculation, following injection of mice with low doses of virus, whereas high doses delayed virus elimination (after i.v. and i.c. infection), and ensured survival following an i.c. inoculation. Animals of both groups expressed virus-specific, T-cell mediated cytotoxicity and antibody production to an equal degree, although, in contrast, DTH reactivity in high-dose mice was absent or severely depressed. Thus, there is an obvious correlation between DTH responsiveness and the efficiency with which the anti-viral immune response reacts to the virus, either in terms of clearance of the infection or induction of immune-mediated disease. Conversely, these experi-

ments and others carried out by differrent groups have unveiled a lack of correlation between a strong Tc response on the one hand and optimal virus clearance and maximal immune-mediated pathology on the other.

Classically, the antimicrobial overall effect of a DTH reaction is explained by the activity of gamma-interferon, migration inhibition factor, macrophage activating factor, lymphotoxins, chemotactic factors, and other lymphokines released from specifically committed Td cells following interaction with the antigen. In turn, attracted mononuclear cells are immobilized and stimulated to phagocytize and destroy the infecting agent. Increased permeability facilitates the influx of antibodies and complement from the blood stream, which again may lead to the appearance of various physiologically active mediators and which also enhances the efficiency of cells bearing Fc and C3 receptors on their surfaces. That this chain of events could be a mechanism of major importance in virus elimination seems, of course, somewhat contradicted by the efficiency and speed with which cloned LCMV-specific Tc cells can reduce the amount of infectious virus in the host and induce fatal immune-mediated disease. On the other hand, the complex DTH reaction may well be highly efficient in assisting Tc function (and antibody) when inoculation is effected with low doses of virus – presumably corresponding to spontaneous infection. Consequently, the adoptive transfer of a relatively large number of highly specialized, cloned T cells into a severely immunosuppressed host may represent an artificial situation in which otherwise meaningful cellular interactions are circumvented and perhaps even impossible.

One could consider the Tc and the Td activity demonstrated in the acute low-dose LCMV infection as two different expressions of the same T-cell response. One explanation of this could be that the acutely sensitized anti-LCMV effector T-cell population consists of cells capable of expressing Tc as well as Td function (Tcd cells), thereby producing the maximal antiviral T-cell response effective in virus elimination and immune-mediated pathology. In general, the Td arm of the cell-mediated immune reaction is the more easily suppressed, and, accordingly, DTH reactivity of a Td cell could be assumed to be preferentially abrogated when high doses of virus are introduced into the mouse, while the Tc function, being more resistant to antigen-induced suppression, prevails.

Alternatively, it could be assumed that only some of the T-effector cells (derived from a common bipotent precursor pool), induced in response to an optimally sensitizing LCMV infection, have received the signal stimulating them to express bifunctionality in terms of cytotoxicity and DTH reactivity, while others – perhaps the majority – only express the more basic Tc function. These models are by no means improbable in light of the several known multifunctional T-cell clones that can change their functional expression with time.

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Regulation and Role of Large Granular Lymphocytes in Arenavirus Infections*

R.M. WELSH

1 Introduction

Cytotoxic lymphocytes have relatively large cytoplasms containing azurophilic granules and are frequently referred to as large granular lymphocytes, or LGL (TIMONEN and SAKSELA 1980). The granules appear to contain cytotoxic substances that are released upon binding of effector cell to target cell (MALLARD et al. 1981). These attach to the membrane of the target cell, and, by uncertain mechanisms, induce lysis of the target (MALLARD et al. 1984; PODACK and DENNERT 1983; HISERODT et al. 1983; WRIGHT et al. 1981). Both natural killer (NK) cells (TIMONEN and SAKSELA 1980; LUINI et al. 1981; REYNOLDS et al. 1981) and cytotoxic T lymphocytes (CTL) (BIRON et al. 1986a) bear LGL morphology, but in the healthy animal the great majority of LGL appear to be NK cells. NK cells are thymus-independent bone marrow-derived lymphocytes that have the ability to lyse on contact a variety of target cells via ill-defined receptors and with no known specificity or antigenic memory (reviewed by WELSH 1984).

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These are distinguished from CTL, which are thymus-derived lymphocytes having T-cell receptor gene rearrangement and expression, conferring a high level of specificity in the killing process. Resting T cells have the morphology of small lymphocytes with unremarkable cytoplasms, but the CTL that are activated during the course of a virus infection develop LGL morphology (BIRON et al. 1986a). As a result, most of the LGL found at later stages in a virus infection belong to the T-cell phenotype. Much of what we know about NK cells and CTL is based on work in arenavirus systems, particularly that of the lymphocytic choriomeningitis virus (LCMV) infection of mice. The characterization, regulation, and potential antiviral properties of cytotoxic lymphocytes induced during arenavirus infections will be the subject of this review. As the roles of T cells in arenavirus infections are discussed in another review in this volume, the major emphasis here will be on NK cells.

2 Historical Background

Early work by TRAUB (1935, 1936, 1938, 1960), ROWE (1954; ROWE et al. 1963), and HOTCHIN (1971; HOTCHIN and BENSON 1963; HOTCHIN and CINITS 1958; HOTCHIN and WEIGAND 1961) indicated that infection of mice with LCMV in utero or at birth usually resulted in a life-long persistent infection. Intraperitoneal (i.p.) or intravenous (i.v.) infection of adult mice usually resulted in clearance of virus with lasting immunity, whereas intracerebral (i.c.) inoculation resulted in a lethal leptomeningitis associated with virus replication in the brain and an intense mononuclear cell infiltrate. Death and the associated meningitis were blocked if those mice were treated with thymectomy or immunosuppressive irradiation or drugs, leading to the suggestion that the disease was mediated by the immune response (ROWE 1954; HAAS and STEWART 1956; ROWE et al. 1963). Adult mice infected by the i.p. or i.v. routes were thought to resist disease because critical target cells in the brain were not infected. The persistently infected mice were thought to resist disease because they failed to mount a significant immune response against the virus (TRAUB 1960) due to tolerance (HOTCHIN and WEIGAND 1961) that may have arisen because the immune system was exposed to viral antigens before it had matured sufficiently to distinguish self from non-self (BURNET and FENNER 1949). That the persistently infected mice were not completely tolerant to LCMV antigens was documented by OLDSTONE and DIXON (1967, 1969, 1970), who showed that LCMV traveled in the circulation of these mice in infectious virus-antibody-complement (C′) complexes, which collected in renal glomeruli, resulting in glomerulonephritis. Thus, the acute LCMV disease was thought to be mediated by a strong immunological response, whereas the persistent LCMV disease was thought to be mediated by a lower level chronic immune response.

Subsequent investigations supported the hypothesis that the pathology in the acute disease was mediated by cellular immunity (VOLKERT and LUNDSTEDT 1968, 1971; TOSOLINI and MIMS 1971; COLE et al. 1972, 1973; GILDEN et al. 1972). MARKER and VOLKERT (1973) documented the presence of LCMV-specific

cytotoxic leukocytes whose activity peaked at day 7 postinfection of adult mice and correlated temporally with the clinical symptoms. COLE et al. (1973) showed that these leukocytes were probably T cells, as the cytotoxicity was eliminated by an antibody to theta, a T-cell antigen, and complement (C′). These investigators also showed that transfer of theta-bearing leukocytes from mice infected 8 days previously with LCMV into acutely infected adult mice immunosuppressed with cyclophosphamide resulted in fatal meningitis (COLE et al. 1972; GILDEN et al. 1972). This led to conclusions that the pathology was T-cell dependent.

Zinkernagel and Doherty next made their revolutionary observations that T-cell dependent functions were restricted to histocompatibility (H-2) antigen determinants and that LCMV-specific CTL recognized LCMV antigens in association with class I (K or D) H-2 determinants. This class I restriction was observed both in cytotoxicity assays (ZINKERNAGEL and DOHERTY 1974, 1975) and in the transfer of cells inducing meningitis using the cyclophosphamide-treated recipient model (DOHERTY et al. 1976). A similar H-2 restriction was shown using a footpad response, in which immune leukocytes were transferred into mice infected with LCMV in the footpad (ZINKERNAGEL 1976). Swelling of the footpad is a result of leukocyte recruitment into a typical delayed-type hypersensitivity lesion (HOTCHIN and BENSON 1963; TOSOLINI and MIMS 1971). Adoptive transfer of immune leukocytes into mice 1 day postinfection with LCMV resulted in up to a 4 $\log_{10}$ decrease in the plaque-forming unit (PFU) titers of virus in the spleen by day 1 posttransfer (MIMS and BLANDEN 1972; ZINKERNAGEL and WELSH 1976). Again, H-2K or D compatibility between donor and recipient was required for this transfer to be effective. These experiments collectively showed that the same type of T-cell recognition patterns and possibly the same type of T cells mediated both immunity and immunopathology in this system.

During the course of examining cytotoxic responses induced by the LCMV infection, responses other than virus-specific were documented. Originally these were dismissed as "background," though two reports suggested that they might be autoreactive or alloreactive CTL (PFIZENMAIER et al. 1975, 1976). Further investigations revealed two discrete peaks of cytotoxic leukocytes induced by the LCMV infection (WELSH and ZINKERNAGEL 1977; WELSH 1978a). A peak at day 7 postinfection corresponded to the well-described, H-2-restricted, virus-specific CTL response, but an earlier peak at day 3 postinfection featured cytotoxic cells which lysed virtually any cultured cell type to which they were exposed. These "early cytotoxic cells" (WELSH 1978a) lysed LCMV-infected and uninfected syngeneic targets equally well, and also lysed allogeneic and xenogeneic targets, as well as targets not expressing any H-2 determinants. Immunochemical characterization of these effector cells revealed similarities between these cells and NK cells, which had just recently been described (KIESSLING et al. 1975a, b; HERBERMAN et al. 1975a, b). The main difference between these early cytotoxic cells and the NK cells previously described was that the NK cells lysed a restricted range of targets, notably T-cell lymphomas, whereas the LCMV-induced cells had an expanded target range and magnitude of lytic activity. This led to the conclusion that the early cytotoxic cells induced by

the LCMV infection were "activated" NK cells, in contrast to the "endogenous" NK cells found in unmanipulated mice (WELSH 1978a, b). Subsequent analyses indicated that the appearance of these activated NK cells correlated with the levels of virus-induced interferon (IFN) type I in the spleen and that IFN activated NK cells when injected directly into mice (WELSH 1978a; GIDLUND et al. 1978). With this historical background, I will now describe what we know of the dynamics of the cytotoxic lymphocyte response to the LCMV infection.

3 Cytotoxic Cell Response to Acute LCMV Infection of Mice

Infection of C3H/St mice i.p. with about 5×10^4 PFU of LCMV Armstrong results, as stated earlier, in a peak in the NK-cell response in the spleen at day 3 postinfection and a peak in the virus-specific CTL response at day 7 postinfection (WELSH and ZINKERNAGEL 1977; WELSH 1978a). Similar responses are seen in other strains of mice (HERBERMAN et al. 1977; WELSH et al. 1979) as well as when utilizing another arenavirus, Pichinde (WELSH 1978a; WALKER et al. 1984). Varying the dose of virus can have an effect on the kinetics of the response, with high doses of virus resulting in peaks of NK-cell activation at earlier times (R.M. WELSH, unpublished). LEHMANN-GRUBE et al. (1981) have shown using the WE strain of LCMV that high doses of virus stimulate NK cell-like activity and relatively low CTL levels, whereas lower doses of virus stimulate lower NK cell-like activity and higher CTL levels. In general, the high levels of NK-cell activation at high doses of virus may relate to the fact that higher doses of virus induce higher levels of IFN, which activates the NK cells. The decreased T-cell response under conditions of high virus doses may be associated with virus-induced immunosuppression resulting from very high levels of virus replication. IFN could also be involved in this immunosuppression. Interestingly, it was observed earlier that very high doses of LCMV injected i.c. failed to induce a fatal meningitis, but instead initiated a persistent infection (HOTCHIN and BENSON 1963). This phenomenon was termed "high dose immune paralysis" (HDIP) and may have been due to the fact that these mice did not develop a sufficient T-cell response to the infection.

The phenotype of mouse NK cells is generally considered to be nylon-wool nonadherent, asialo GM1$^+$, NK 1.2 alloantigen$^+$, Qa-5$^+$, Lyt 1$^-$, Lyt 2$^-$, Lyt 5$^+$, L3T4$^-$, Mac-1$^+$, Ia$^-$, and Thy 1.2$^{+/-}$ (reviewed in WELSH 1984; WELSH et al. 1984). The morphology is that of an LGL. The phenotype of the CTL is nylon-wool nonadherent, asialo GM1$^{+/-}$, NK 1.2$^-$, Qa-5$^+$, Lyt-1$^{+/-}$, Lyt 2$^+$, Lyt 5$^+$, L3T4$^-$, Mac-1$^-$, Ia$^-$, and Thy 1.2$^+$. Upon activation, the CTL, like the NK cell, has the morphology of an LGL (BIRON et al. 1986a). Hence, there are both similarities and differences in the phenotypes of NK cells and T cells.

3.1 NK-Cell Activation and Proliferation

In addition to their ability to kill a broader spectrum of target cells, the isolated NK cells at 3 days post-LCMV infection have a slightly different phenotype

than endogenous NK cells. They are more adherent to nylon wool, express more Fc receptor-mediated adherence, and have a slightly enhanced sensitivity to depletion with antibody to Thy 1.2 and C′ (KIESSLING et al. 1980; HERBERMAN et al. 1978). They also appear to be slightly more resistant to treatment with antibodies to asialo GM1 (YANG et al. 1985), NK 1.2 (KUMAR et al. 1982), or Lyt 5 (YANG et al. 1985), and C′. These differences are relatively minor, and the activated NK cells still can be distinguished from CTL, though not as clearly as can the endogenous NK cell. An in-depth study by YANG et al. (1985) suggested that the apparent resistance to depletion with antibodies to asialo GM1 was more likely due to the fact that splenocytes from LCMV-infected mice express much higher levels of asialo GM1 than do those from uninfected mice, and they thus compete for the antibody. Analyses by flow cytometry indicated that all the NK-cell activity present at day 3 postinfection was mediated by cells expressing very high levels of asialo GM1 (YANG et al. 1985). What differences do exist between the activated and the endogenous NK cells appear to be due to two phenomena: changes in the surface antigen expression due to activation and changes in the size of the cell due to proliferation. In fact, the augmented NK-cell response seen during the LCMV infection is a function of both the activation (WELSH 1978a) and very significant levels of proliferation (BIRON and WELSH 1982; BIRON et al. 1983) of the NK-cell population.

Activated NK cells have been shown in a variety of systems to have an expanded target-cell range (WELSH and ZINKERNAGEL 1977; NUNN et al. 1977), more rapid kinetics of lysis (TARGAN and DOREY 1980; ULLBERG and JONDAL 1981), and the ability to kill a second or third time (recycling) (ULLBERG and JONDAL 1981; TARGAN 1981). All of these properties are stimulated by IFN, although other substances, such as interleukin-2 (Il-2) can also stimulate them (KURIBAYASHI et al. 1981). NK cells release cytotoxic factors which bind to the surface of target cells and mediate lysis of those targets (WRIGHT et al. 1981; HISERODT et al. 1983). These factors, called NK-cell cytotoxic factors, or NKCF, are released from NK cells upon exposure to appropriate lectins or target cells (WRIGHT and BONAVIDA 1981, 1983a). IFN-treated NK cells release more of these factors than do unstimulated NK cells (WRIGHT and BONAVIDA 1983a).

The kinetics of the NK-cell response in the LCMV infection very closely parallel the IFN-type I levels in the spleen (WELSH 1978a), and in mice infected with Newcastle disease virus an antibody to IFN blocks the activated NK-cell response (GIDLUND et al. 1978). These results would indicate that IFN is the predominant factor regulating NK-cell activity during virus infections. The NK-cell response declines as the CTL response becomes detectable (WELSH and ZINKERNAGEL 1977). This is likely due to the fact that the virus titers drop as a result of T-cell surveillance, and the IFN levels decline because the inducing agent (LCMV) has disappeared. Athymic nude mice, which have no detectable CTL and maintain high titers of LCMV PFU and LCMV-induced IFN for prolonged time periods, also maintain activated NK cells for prolonged periods (WELSH 1978a).

NK cells in unmanipulated mice are normally medium-sized lymphocytes, but during LCMV infection, the size distribution shifts to large, blast-sized cells, which can be detected by centrifugal elutriation (KIESSLING et al. 1980;

Biron and Welsh 1982). Several experiments confirmed that these were indeed blast NK cells. LCMV infection of irradiated mice stimulated activation of the small- to medium-sized NK cells, as shown by the enhanced levels of cytotoxicity against target cells, but no NK-cell–mediated lysis was found in large, blast-size cell fractions (Biron and Welsh 1982). This suggested that although activation of NK cells can occur without cell division (Welsh 1978a), cell division must be required for the appearance of the large NK cells. Freshly isolated leukocytes were given a 1-h pulse of [^{3}H]thymidine and incorporated into a single-cell cytotoxicity assay in agarose (Biron and Welsh 1982). Both K562 and YAC-1 target cells were used. The suspensions were incubated and the dead cells stained with trypan blue. After fixation with formalin, the preparations were exposed to photographic emulsions for autoradiography. The results indicated that less than 3% of the endogenous NK cells lysing targets had incorporated the thymidine label, but 20% of the LCMV-induced NK cells incorporated thymidine. Furthermore, the thymidine-incorporating killer cells were located in the blast-size fractions after separation by centrifugal elutriation. This suggested that the spleen NK-cell population was a resting cell population converted into division by the virus infection. Treatments of mice with hydroxyurea, a drug which kills dividing cells, had little effect on endogenous NK-cell activity, but profoundly depleted the virus-induced activity (Biron et al. 1983). This drug also reduced the number of thymidine-incorporating NK cells after LCMV infection. These experiments therefore indicate that the LCMV infection not only causes activation of the NK-cell population in terms of cytotoxic properties, but it also converts a relatively resting cell population into one which is rapidly turning over and undergoing division. Recent work indicates that these dividing NK cells can also be found in the liver and peritoneum after infection (C.A. Biron, K.W. McIntyre, R.J. Natuk, unpublished).

The peak in NK-cell division correlates with the peak in NK-cell activation, and, consequently, the peak in the IFN response (Biron and Welsh 1982). To determine whether IFN directly induced NK-cell blastogenesis, mice were injected with the IFN inducer poly inosinic:cytidylic acid (poly I:C), purified IFN beta, or crude (Biron et al. 1984) or recombinant (C.A. Biron, unpublished) IFN gamma. All treatments stimulated the production of blast NK cells. Even further augmentation of NK-cell activity and blastogenesis was seen upon sequential stimulations of mice with poly I:C and/or IFN beta (C.A. Biron, unpublished).

Associated with the increase in NK-cell blastogenesis and turnover in LCMV infection is an apparent increase in NK-cell number. Both the number of LGL (Biron et al. 1983) and the number of cells brightly staining with antibody to asialo GM1 (Yang et al. 1985) increase about three fold in the spleen by day 3 postinfection. Large increases in asialo GM1 positive cells and LGL are also found in the liver and peritoneal cavity by day 3 postinfection (Welsh et al. 1986; Natuk and Welsh 1987; McIntyre and Welsh 1986). These experiments collectively indicate that during the LCMV infection, IFN induces the blastogenesis and consequential proliferation of the NK-cell population.

How IFN works to stimulate NK-cell proliferation is not known. NK cells are *not* stimulated to proliferate in vitro by the simple addition of IFN. In

fact, IFN is more commonly known for its ability to inhibit cell growth than to be mitogenic (GRESSER et al. 1974). Thus, although it is possible than IFN is directly mitogenic to NK cells, this seems unlikely, and a more complicated mechanism is probably involved. IFN may act to induce a growth factor for NK cells or to induce receptors for a growth factor on NK cells. It has been suggested that IFN may induce receptors for IL-2 (FARRAR et al. 1982; KURIBAYASHI et al. 1981; MINATO et al. 1981), and the few "true" NK-cell clones that do exist grow in IL-2–containing medium (RITZ et al. 1985). However, in contrast to T-cell clones, the NK-cell clones cannot be maintained with only recombinant IL-2 and must be cultured with cruder lymphokine supernatants (R. SCHMIDT, personal communication). Highly purified NK 1.1 alloantigen plus mouse NK cells will undergo several generations of proliferation in the presence of recombinant IL-2, but only at concentrations 100 times that required for T-cell growth (M.M. TUTT et al., submitted). Young athymic nude mice, which have not been shown to be able to produce IL-2 in vitro, undergo a normal blastogenic response upon IFN stimulus in vivo (BIRON et al. 1983, 1986b). These data collectively indicate that IL-2 is probably not the only growth factor for NK cells. Perhaps NK cells have their own growth factor and growth factor receptor which bear some homology with IL-2 and the IL-2 receptor, allowing for modest IL-2-dependent effects due to the low-affinity binding of the ligand.

3.2 CTL Response to Infection

The H-2 restricted, virus-specific, Lyt 2^+ CTL response, reviewed elsewhere in this volume, peaks at 7–9 days postinfection (MARKER and VOLKERT 1973; ZINKERNAGEL and DOHERTY 1974). Its appearance coincides with the clearance of virus and with the reduction of IFN and NK-cell levels (MARKER and VOLKERT 1973; ZINKERNAGEL and DOHERTY 1974; WELSH 1978a; BUCHMEIER et al. 1980). During the acute LCMV infection, both the number of cells per spleen and the percentage of Lyt 2^+ spleen leukocytes doubled by day 7 postinfection, resulting in about a four fold increase in cells of the CTL phenotype (BIRON et al. 1986a). Whereas less than 3% of the Lyt 2^+ cells in normal mice have the LGL phenotype, nearly 25% of the Lyt 2^+ cells by day 7 postinfection are LGL (BIRON et al. 1986a). We believe that conversion of T cells to this LGL phenotype indicates the acquisition of cytotoxic function. Thus, a large number of T cells appears to have cytotoxic potential after LCMV infection.

Recent work indicates that not all of these T cells are LCMV specific and that the LCMV infection is actually a potent stimulator of CTL which are apparently not reactive with LCMV antigens. C57BL/6 mice infected with LCMV generate allospecific CTL concomitantly with LCMV-specific CTL (YANG and WELSH 1986a, b). These allospecific CTL could easily be confused with activated NK cells, but they do have a different antigen phenotype and different target specificities. They are most easily detected in mice whose NK cells have been depleted by treatment in vivo with antibody to asialo GM1. The remaining leukocytes lyse allogeneic but not syngeneic or xenogeneic targets and have the phenotype Thy 1.2^+, Lyt 2^+. A possible explanation for this phenomenon is that, to the CTL of a C57BL/6 mouse, virus-modified self may

resemble alloantigens. However, cold-target competition assays showed that the lysis of the allogeneic targets was not inhibited by virus-infected syngeneic targets, suggesting that the effector cells were not cross-reactive between the two target types. These data are more consistent with the concept that LCMV is a polyclonal stimulator of unrelated CTL types.

Other systems support the concept of arenaviruses being polyclonal CTL stimulators. Treatment of mice with cyclophosphamide before PV infection abrogates the primary CTL response, and when these mice receive a PV challenge several weeks later, no secondary PV-specific memory CTL response is detected (WALKER et al. 1985). However, if these mice are coinfected with PV and LCMV or with PV and Tacaribe virus, they develop PV-specific memory CTL. This suggests that the second virus either provided help or eliminated suppression which allowed for the generation of PV-specific CTL. WALKER et al. (1985) showed that IL-2–containing supernatants could substitute for the heterologous virus in stimulating the PV-specific CTL. This suggests that the lymphokines liberated during the acute primary arenavirus infections may contribute to the activation of other CTL. IL-2 has also been shown to augment allospecific CTL responses when injected into mice along with alloantigens (HEFENEIDER et al. 1983). Mice hyperimmunized with two injections of LCMV produce potent LCMV-specific CTL responses, which decline to undetectable levels several days postinfection. Challenge of these mice with PV, Murine Cytomegalovirus (MCMV), or vaccinia virus not only stimulates CTL against the challenge virus, but also against LCMV (YANG and WELSH 1986b). This indicates that the activation of apparently irrelevant CTL responses may be a common property of a number of viruses.

3.3 Accumulation of LGL at Sites of Virus Infection

NK-cell number and activity is not only augmented in the spleen but in many other organs as well, including the peritoneal cavity (WELSH 1978a), liver (WELSH et al. 1986), lung (BIRON et al. 1984b), bone marrow (THOMSEN et al. 1986), and peripheral blood (STITZ et al. 1985). The kinetics of the NK-cell response are not necessarily identical to those in the spleen. NK activity in the peritoneum, liver, and, in particular, peripheral blood remains somewhat elevated through day 7 postinfection. Not only is the activity of the NK-cell population enhanced in these organs, but there is also an elevation in the number of LGL with the NK-cell phenotype. In this respect, both the peritoneal cavity and the liver have shown similar responses (MCINTYRE and WELSH 1986; NATUK and WELSH 1987). Under normal conditions less than 1% of the leukocytes in the peritoneal cavity and about 5%–7% of the leukocytes in the liver are LGL. Three days after LCMV infection, 3%–5% of the peritoneal leukocytes and 10%–12% of the liver leukocytes are LGL. Similar results in the peritoneal cavity are seen in PV-infected mice. Less than 1% of the leukocytes in these virus-infected mice are LGL if the mice are first treated with antibody to asialo GM1, correlating with the complete elimination of NK-cell activity. At day 7 postinfection, when the CTL activity is peaking, there is a tripling of the percentage of LGL

(compared with day 3) along with a massive (5- to 20-fold) influx of leukocytes into the peritoneal cavity (BIRON et al. 1986a; WELSH et al. 1986; NATUK and WELSH 1987). This influx is also seen in the livers of mice infected with the hepatotropic WE strain of LCMV but not with the nonhepatotropic Armstrong strain (WELSH et al. 1986; MCINTYRE and WELSH 1986). The net result is that there is a 20- to 100-fold increase of LGL between day 3 and day 7 postinfection. This influx in cell number is probably T-cell dependent, as it does not occur in athymic nude mice (WELSH and DOE 1980; MCINTYRE and WELSH 1986), and the LGL present at day 7 include many CTL-LGL. Whereas antibody to asialo GM1 completely eliminates the LGL present at day 3, it only partially reduces the LGL present at day 7 (BIRON et al. 1986a). Treatment of these leukocytes with antibody to Lyt 2 plus C′ in vitro reduces the LGL number (MCINTYRE and WELSH 1986). Further, high levels of virus-specific, H-2–restricted CTL activity are associated with these cell populations.

Accumulation of NK cells at the site of virus infection is likely to be at least partially due to chemotaxis, which has recently been demonstrated for both human and rat NK cells (BOTTAZZI et al. 1985; POHAJDAK et al. 1986). Peritoneal fluid from virus-infected mice is chemotactic to NK cells in vitro (NATUK and WELSH 1987). Where the NK cells come from just before accumulation at these sites of infection is not known. Although the spleen functions as a major organ of NK-cell division during infection (BIRON and WELSH 1982), splenectomy (during mouse hepatitis virus infection) only results in marginal reductions in liver or peritoneal LGL (K. MCINTYRE and R. NATUK, unpublished data). It is thus likely that the NK cells come from another organ, such as the bone marrow, which is known to be the ultimate source of NK cells and their precursors (HALLER et al. 1977; HALLER and WIGZELL 1977). Some of the increase in the organ-associated NK-cell number may be due to in situ proliferation. Mitotic figures can be seen in liver and peritoneal LGL, and [^{3}H]thymidine-incorporating leukocytes from the liver and peritoneal cavity lyse target cells in single-cell cytotoxicity assays (C.A. BIRON, K.W. MCINTYRE, and R.J. NATUK, unpublished).

3.4 Secondary Stimulation of Cytotoxic Lymphocytes

Secondary stimulation of LCMV- or PV-immune mice with homologous virus results in a memory CTL response peaking earlier (day 4 postinfection) than in primary infections (LEHMANN-GRUBE et al. 1981; WALKER et al. 1984, 1985). The magnitude of the secondary CTL response is often less than the acute response, most likely because the virus is cleared so rapidly. It was originally reported that LCMV infection of LCMV-immune mice did not stimulate an activated NK-cell response, whereas PV infection did (WELSH 1978a). However, the only time point examined was day 3 postinfection, which is the peak of NK activity during the acute response. More recent work indicates that PV infection of PV-immune mice activates NK cells at day 1 postinfection, but the activity rapidly declines thereafter (WALKER et al. 1984). The most likely

explanation for this is that the initial waves of virus replication induce enough IFN to activate the NK cells. The rapid memory CTL and/or antibody response then quickly clears the virus before more IFN is induced to continue the NK-cell activation. IFN-gamma or IL-2 produced by the primed T cells could also possibly contribute to this NK-cell response.

It has been suggested that the secondary NK-cell response seen during PV infection may represent a "memory" NK-cell response (WALKER et al. 1984). There are at least three possible mechanisms by which a memory NK-cell response could arise:

1. IFN-induced proliferation or priming of the NK-cell population may result in an even greater NK-cell response upon a second IFN stimulus, providing the NK-cell number and IFN-induced effects had not declined to normal levels
2. Factors such as IFN-gamma and IL-2 produced by memory T cells may serve to activate NK cells in a rapid and effective fashion
3. The NK cells themselves may have antigenic specificity and clonally expand during infection.

This latter point deserves some comment, as it has even been suggested that the NK cells activated during infection may be precursors to the virus-specific CTL (WALKER et al. 1984). This suggestion was partially based on reports that Thy 1.2 antigen increases on LCMV-induced activated NK cells during virus infections (HERBERMAN et al. 1978; KIESSLING et al. 1980) and is given further credence by reports that certain cloned cells lines with NK-cell activity have rearrangements and/or expression of T-cell receptor genes (RITZ et al. 1985; YANAGI et al. 1985). If this were the case, then one might predict that NK cells do have the potential for specificity. However, recent work with freshly isolated and purified human NK cells (LANIER et al. 1986), with $T3^-$ human NK-cell clones (RITZ et al. 1985), with a rat NK-cell tumor (REYNOLDS et al. 1985), and with NK 1.1^+ mouse NK cells purified by flow cytometry and expanded in culture with IL-2 (TUTT et al. 1986) indicate that NK cells do not have T-cell receptor gene rearrangements or expression of functional transcripts. Analysis of the T-cell receptor genes is clearly needed for the freshly isolated activated NK cells that are stimulated during virus infection. However, we believe that it is very unlikely that the activated NK cells represent CTL precursors for several other reasons: normal CTL responses occur in mice whose NK cells have been depleted with ^{89}strontium (WELSH 1978a) or with antibody to asialo GM1 (YANG and WELSH 1986a), purified blast NK cells from LCMV infected mice do not generate into LCMV-specific CTL in limiting dilution assays in vitro (BIRON and WELSH 1986), and LCMV-specific CTL precursors, as well as the precursors for other CTL, are Lyt 2^+, an antigen not found on the activated NK cells (BIRON and WELSH 1986). Whether arenavirus-specific memory NK cells exist is actually not supported by convincing data. Although the peak in NK activity during a secondary PV response is at day 1 instead of day 3 postinfection, the magnitude of the secondary response is no greater than at day 1 during the acute response (WALKER et al. 1984). Whereas some mechanisms may exist to allow for a memorylike response for NK cells, it is unlikely

to be a memory response in the true immunological sense, and good evidence for any kind of memory NK-cell response during arenavirus infections is lacking.

3.5 Cytotoxic Cells in Persistently Infected Mice

Mice persistently infected with LCMV by congenital infection or neonatal inoculation do not develop a detectable CTL response and can be considered "tolerant" at the level of CTL. Why CTL are not detectable is the subject of much controversy and research and will not be reviewed here. These mice, of course, are not completely tolerant to LCMV, as they make antibody responses to all the LCMV structural proteins (OLDSTONE and DIXON 1967; 1969; BUCHMEIER et al. 1978).

The persistently infected mice have a chronic low level interferonemia, characterized by titers of 8–64 units of IFN/ml blood over periods of many months (SARON et al. 1982; BUKOWSKI et al. 1983b). Correlating with the low levels of IFN are chronically elevated levels of NK-cell–mediated lysis which lasts for many months (BUKOWSKI et al. 1983b). These NK cells are not at the level of activity of NK cells isolated from acute infections. For instance, although lysis is elevated against the NK-sensitive YAC-1 cells, there is no lysis against the relatively resistant L-929 cells (WELSH 1978a; BUKOWSKI et al. 1983b). NK cells from these mice are not found in blast-sized cell fractions unless IFN is further induced by poly I:C (BUKOWSKI et al. 1983b). This suggests that there is not a marked increase in proliferation of NK cells in these animals, but rather a modest increase in their state of activation or cell number.

4 Regulation of Arenavirus Infections by NK Cells

Several lines of evidence indicate that NK cells do not influence LCMV synthesis in vivo. LCMV PFU production is normal during the early stages of infection of mice whose NK-cell activity has been depleted by cyclophosphamide (WELSH et al. 1984) or antibody to asialo GM1 (BUKOWSKI et al. 1983a). There is no genetic basis of resistance in mice to LCMV that correlates with NK-cell activity, and mice bearing the beige mutation, which confers a defect in the cytotoxic capabilities of NK cells (RODER and DUWE 1979), synthesize normal levels of virus (WELSH and KIESSLING 1980). Adoptive transfer of adult leukocytes with high NK-cell activity into 5-day-old suckling mice with low NK-cell activity did not confer any resistance to LCMV, even though it markedly conferred resistance to the NK-cell sensitive virus, MCMV (BUKOWSKI et al. 1985). Depletion of NK-cell activity with antibody to asialo GM1 had no effect on the synthesis of either the WE or the Armstrong strain of LCMV in acute infections or of the Armstrong strain during persistent infection (BUKOWSKI et al. 1983a; WELSH et al. 1984).

NK cells may, however, play a role in murine PV infection. We have consistently noticed a 2- to 3-fold enhancement in PV synthesis at day 3 postinfec-

tion of mice depleted of NK cells by antibody to asialo GM1 (R.J. NATUK and R.M. WELSH, unpublished). Further, in contrast to results with LCMV, adult leukocytes protect suckling mice from PV, and this protection is eliminated if NK cells are depleted from the donor leukocyte population. These apparent anti-PV NK-cell–mediated effects are not as great as those seen with MCMV but are nevertheless in sharp contrast with those seen with LCMV. That two viruses within the same virus group have differential sensitivities to NK cells has also been seen in the herpes virus group. MCMV appears very sensitive to NK cells, but neither herpes simplex virus type 1 (HSV-1) (BUKOWSKI and WELSH 1986a) nor type 2 (HSV-2) (MORAHAN et al. 1982) share this sensitivity. It would be of interest to know whether the differential sensitivities of LCMV and PV to NK cells are traits peculiar to those viruses, or whether they represent differences between New World and Old World arenaviruses. In general, information is lacking concerning the susceptibilities of other arenaviruses to NK cells. Of interest is that immune serum (antibody) therapy is used to treat animals and man infected with Lassa virus (JAHRLING and PETERS 1984) and with Junin virus (MAIZTEGUI et al. 1979), and NK-like cells, particularly in man, bear Fc receptors which enable them to mediate antibody-dependent cell-mediated cytotoxicity (ADCC) against a variety of infections (SISSONS and OLDSTONE 1980). Neutralizing antibody seems to be required for optimal serum therapy against Lassa fever, suggesting that ADCC may not be the primary mechanism of therapy (JAHRLING and PETERS 1984), but it would be of great interest to examine the role of ADCC, particularly in the New World hemorrhagic fever virus infections. At present, however, very little is known about NK cells in human arenavirus infections.

Preparations of LCMV glycoproteins appear to activate human NK cells in vitro (CASALI et al. 1981). In vitro activations of human natural cytotoxicity have also been noted with glycoproteins isolated from measles (CASALI et al. 1981), mumps (HARFAST et al. 1980), and Sendai (ALSHEIKHLY et al. 1983) viruses. Extensive analyses in the mumps and Sendai virus systems have, however, indicated that the major effector cell in this form of cytotoxicity is a T cell and not an NK cell (ALSHEIKHLY et al. 1985), suggesting that further analyses may be needed for the effector cell activated by LCMV.

Little is known about the replication of viruses in NK cells (CASALI et al. 1984), but it has been suggested that NK cells in the hamster may be target cells for PV replication and that hamsters having high NK activity may therefore be more susceptible to infection (GEE et al. 1979). MHA hamsters have high NK activity and replicate high levels of PV, whereas LSH hamsters have low NK activity and replicate low levels of PV. When leukocytes were placed on velocity gradients, the peak in virus titers correlated with the peak in NK-cell activity on those gradients (GEE et al. 1979). Inconsistent with this hypothesis is that PV grows better in baby hamsters or in cyclophosphamide-treated hamsters, both of which lack NK activity. The association between high NK-cell activity and high titers of virus may also have reflected the fact that the high titers of virus stimulate high levels of IFN, which stimulates high levels of NK-cell activity. It is nevertheless possible that PV does grow in hamster NK cells, but more work is needed to support this hypothesis.

5 Regulation of Arenavirus Infections by CTL

Evidence that CTL regulate the synthesis of LCMV in infected mice is derived mostly from experiments utilizing adoptive transfers of LCMV-immune leukocytes into recipient mice at days 1–3 after acute infection and examining virus titers in the organs 1–2 days thereafter (MIMS and BLANDEN 1972). Cells with the cytotoxic phenotype mediate clearance of the virus. Adoptive immunization is mediated by theta or Thy 1.2^+, Lyt 2^+ lymphocytes requiring H-2K or D but not I compatibility with recipient mice (ZINKERNAGEL and WELSH 1976; VARHO et al. 1981; LEHMANN-GRUBE et al. 1985). Acutely infected mice can also be protected by LCMV-specific CTL clones in an H-2–restricted manner (BYRNE and OLDSTONE 1984). Whereas there is concensus on this point, it remains an issue whether the CTL mediate resistance by lysing virus-infected targets or whether they mediate resistance indirectly by secreting lymphokines and involving other cells of the immune system. LEHMANN-GRUBE et al. (1985) have calculated that very low numbers of effector cells actually get into the spleen during adoptive transfers, yet the virus in the spleen is cleared with remarkable efficiency. They suggest that the virus may even be directly inactivated. T cells are known to produce a variety of lymphokines, including IFN gamma, and influenza virus-specific CTL clones produce IFN gamma shortly after exposure to appropriate virus-infected target cells (MORRIS et al. 1982). IFN gamma not only can mediate a direct antiviral effect, but it can also induce NK-cell activation (KUMAR et al. 1979) and proliferation (BIRON et al. 1984) and macrophage activation (SCHULTZ and KLEINSCHMIDT 1983).

Macrophage activation factor (MAF) is now thought to be an activity mediated by IFN gamma (SCHULTZ and KLEINSCHMIDT 1983). LCMV-specific T cells with CTL (H-2K or D) recognition patterns can stimulate delayed-type hypersensitivity (DTH) reactions, as noted by the footpad response, which are characterized by the accumulation of activated macrophages (ZINKERNAGEL 1976). It can thus be surmised that the CTL do secrete IFN gamma and possibly other lymphokines into their environment in the infected mouse. These facts attest to the likelihood that the CTL may not mediate their effects via direct cytotoxicity. However, adoptive transfer of LCMV- or PV-specific spleen cells into recipient mice coinfected with both LCMV and PV reduced the titers only of the virus to which the T cells were primed (MCINTYRE et al. 1985). Similar results were found in irradiated mice infected for 3 days before leukocyte transfer.

Immunofluorescent staining showed LCMV- and PV-infected cells in proximity to each other before the cell transfer. This experiment supports the hypothesis that the adoptive immunization is mediated either by a cytotoxic event or by a very restricted secretion of soluble factors, rather than a more generalized nonspecific antiviral event that would be expected to be mediated by IFN gamma. Similar conclusions were drawn in the influenza system using two strains of influenza virus and influenza strain-specific CTL clones (LUKACHER et al. 1984). Thus, the data suggest that LCMV-specific CTL regulate the LCMV infection and that they may do so at least in part by direct cytotoxic mechanisms.

6 Effect of IFN on Target-Cell Susceptibility to NK Cells and CTL

Arenavirus infections are potent stimulators of IFN in vivo (PADNOS et al. 1971; BRO-JORGENSON and KNUDTZON 1977; RIVIERE and BANDU 1977; MERIGAN et al. 1977), and treatment of mice with antibody to IFN enhances LCMV synthesis (GRESSER et al. 1978). Most of the IFN found in the spleen of LCMV-infected mice is type I (α or β), but in vitro culture of leukocytes harvested from the spleen results in the production of high levels of IFN gamma (WELSH and DOE 1980). The IFN induced during the infection has marked effects on cells in the body. Infection of mice with the Pasteur strain of LCMV results in severe abnormalities of the liver and kidney, which can be blocked by antibody to IFN (RIVIERE et al. 1977). Further, treatment of adult mice infected with an aggressive neurovirulent strain of LCMV with antibody to IFN alters the course of infection and converts the syndrome to a prolonged, less severe infection as seen with a "docile" strain of LCMV (PFAU et al. 1983).

One of the properties of IFN is its ability to induce the expression of H-2 antigens on the surfaces of treated cells (LINDAHL et al. 1976). Thymocytes and bone-marrow cells isolated from LCMV-infected mice 3 days postinfection express five to tenfold greater levels of class I H-2 antigens on their surfaces (BUKOWSKI and WELSH 1986b). This is likely due to an IFN effect, as high levels of IFN are produced at that time, and this effect can be mimicked, though not quite as effectively, with injections of purified IFN beta or with poly I:C. Another effect of IFN is its ability to alter glycosphingolipid metabolism and to enhance the expression of cell surface sialic acid (YOGEESWARAN et al. 1982, 1983). Splenocytes isolated from mice acutely infected with LCMV likewise have up to fourfold increases in the expression of sialic acid, neutral glycosphingolipids, and gangliosides (YANG et al. 1985). Thus, the animal, during its response to virus infections, undergoes marked biochemical alterations.

The bone-marrow cells and thymocytes isolated from LCMV-infected mice are markedly more susceptible than untreated controls to lysis by allospecific CTL (BUKOWSKI and WELSH 1986b). This correlates with their heightened expression of class I H-2 antigens, which are the target structures for CTL. An unusual additional property of IFN is its ability to protect target cells from NK-cell mediated lysis (TRINCHIERI and SANTOLI 1978; MOORE et al. 1980; WELSH et al. 1981). NK cells bind to IFN-treated targets (PERUSSIA and TRINCHIERI 1981; WELSH et al. 1981), but fail to release the cytotoxic factors which lyse the target cell (WRIGHT and BONAVIDA 1983b). IFN-treated targets do not compete as cold targets against the lysis of normal target cells, and this is likely because the NK cells do not discharge their granules and become inactivated (PERUSSIA and TRINCHIERI 1981). H-2–negative cell lines are not protected by IFN, even though they are susceptible to the antiviral and growth inhibitory effects of IFN (PIONTEK et al. 1985). This has led to speculations that NK cells may recognize or be triggered by a "lack of H-2", whereas CTL recognize H-2 (PIONTEK et al. 1985; KARRE et al. 1986). Consistent with this hypothesis is that thymocytes from LCMV-infected mice express high levels of H-2 antigens and high sensitivity to lysis by allospecific CTL, but are markedly less sensitive to lysis by activated NK cells (HANSSON et al. 1980). This does not appear to

be due to selection against the NK-sensitive thymocytes in vivo, as poly I:C stimulation of beige mice, which have poor NK activity, also results in resistant thymocytes. Further, treatment of thymocytes with IFN in vitro renders them resistant to LCMV-induced activated NK cells (HANSSON et al. 1980). Similarly, tumor cells grown as ascites in either beige or normal mice infected with LCMV become resistant to NK-cell mediated lysis when tested in vitro (WELSH et al. 1981). That IFN protection against NK cells may be functioning in vivo is attested to by the fact that radiolabeled IFN-treated tumor cells resist clearance (in vivo lysis) when injected i.v. into mice (WELSH et al. 1981).

The effects of IFN on target cells may be of importance in determining the sensitivity of LCMV-infected targets to NK cells and to CTL. Exposure of LCMV-infected embryonic fibroblasts to either IFN beta or gamma protects them from lysis by LCMV-induced activated NK cells, but renders them two to four times more susceptible to LCMV-specific or to allospecific CTL (BUKOWSKI and WELSH 1985a, b, 1986b submitted). Continuous cell lines such as L-929 and MC57G are also protected from NK cells, but are not rendered substantially more susceptible to CTL-mediated lysis (BUKOWSKI and WELSH 1985a). These continous cell lines, however, express very high levels of H-2 antigens, which are not further elevated after exposure to IFN (BUKOWSKI and WELSH 1985a). The fibroblasts, as well as many of the cells and organs in the body, normally express relatively low levels of H-2, and exposure of these to IFN in vitro or in vivo greatly enhances their expression of H-2 (BUKOWSKI and WELSH 1985a; LINDAHL et al. 1976; SKOSKIEWICZ et al. 1985). These IFN-mediated effects may thus explain why CTL but not NK cells regulate the LCMV infection. One would hypothesize that during the LCMV infection both uninfected and LCMV-infected target cells become protected by IFN as the NK cells become activated and proliferate. Whereas IFN by itself may inhibit LCMV synthesis (GRESSER et al. 1978), it would not do so by an NK cell-dependent mechanism. Of interest is that IFN does not protect target cells infected with the NK cell-sensitive MCMV from NK cell-mediated lysis (BUKOWSKI and WELSH 1985b). Later in the infection, as the CTL develop and expand in number, the IFN-treated virus-infected target cells become ideal targets for CTL by virtue of their increased expression of H-2 antigens (BUKOWSKI and WELSH 1985a). These CTL would then clear the infection.

Similar mechanisms could also possibly contribute to the immunopathology associated with the acute i.c. LCMV infection. Pfau and coworkers (PFAU et al. 1982, 1983, 1985; JACOBSON et al. 1981) have documented distinct pathways of immunopathology in mice infected with either of two strains of LCMV, termed docile and aggressive. The aggressive strain induces high levels of IFN, replicates to relatively low levels in the viscera, and develops a severe meningeal inflammatory cell reponse that results in the classic leptomeningitis associated with normal i.c. LCMV infections. Mice infected with the docile strain produce low levels of IFN, replicate high levels of virus in the viscera, and have a more prolonged disease syndrome not associated with acute meningitis (PFAU et al. 1982; JACOBSON et al. 1981). Both infections result in comparable CTL responses, which can be found in both spleen and cerebrospinal fluid (PFAU et al. 1985). IFN inducers such as poly I:C convert the docile infection into

an aggressive one (JACOBSON et al. 1981), and antibody to IFN converts the aggressive infection into a docile one (PFAU et al. 1983). In these studies the presence of high levels of IFN was associated with reduced levels of virus in the visceral organs, leading Pfau and coworkers to hypothesize that the high levels of virus replication in the viscera may provide an "antigen sink" which inhibits the flow of the appropriate T cells to the brain (PFAU et al. 1982). This is an appealing hypothesis, but another factor that could be involved is that the high levels of IFN may condition the cells in the meninges to be more susceptible to attack by immune T cells, via the mechanism of enhanced H-2 expression, as discussed above. It would be interesting to test this theory in this system.

7 Potential Consequences of NK-Cell Activation

NK cells, of course, have functions other than mediating resistance to viruses, and the potent augmentation that one sees during arenavirus infections may affect those functions.

7.1 Antitumor Effects

Mice acutely infected with LCMV resist tumor formation by implanted tumor cells (MOLOMUT and PADNOS 1965) and reject 125-IUDR-labeled, i.v. injected tumor cells is short-term in vivo cytotoxicity assays (TALMADGE et al. 1980; BIRON et al. 1984b). The impairment of tumor growth and the rejection of the labeled tumor cells are probably both due to NK-cell effects. The inhibition of tumor growth occurs in athymic nude mice (WELSH, unpublished) but is depressed in NK-cell deficient beige mice (TALMADGE et al. 1980). The rejection of implanted [^{125}I]UDR-labeled cells, well characterized to be mediated by NK cells in other systems (RICCARDI et al. 1979, 1981), is blocked in the LCMV system by antibody to asialo GM1 (BIRON et al. 1984b) and is reduced in beige mice (TALMADGE et al. 1980). Mice persistently infected with LCMV have moderately elevated levels of NK-cell activity and reject implanted tumor cells more efficiently than do control mice (BUKOWSKI et al. 1983b). These experiments collectively show that the LCMV infection may have antitumor properties by activating NK cells. Of course, induction of tumor-specific T cells could also be augmented but has thus far not been documented in this system. The fact that acute LCMV infection results in the generation of allospecific CTL lends credence to this possibility (YANG and WELSH 1986a). Of interest is that LCMV in the past has been used to treat human malignancies (WEBB et al. 1975). Although this treatment is not currently in use because of the potential neurotropism of LCMV in humans, we now have a clue as to the mechanism behind the therapeutic efficacy.

7.2 Hematopoiesis

Acute LCMV infection causes a marked inhibition of the hematopoietic function of the bone marrow (BRO-JORGENSON 1978). Irradiated LCMV-infected mice are poor recipients of bone-marrow transfers, as measured by spleen colony-forming units (CFUs), and bone-marrow cells from LCMV-infected mice when transferred into LCMV-immune or LCMV-carrier irradiated mouse recipients are deficient in giving rise to CFUs. The deficiency in CFU formation correlates temporally with IFN production (BRO-JORGENSON 1978; BRO-JORGENSON and KNUDTZON 1977), and, consequently, the activation of NK cells. The inhibition of hematopoiesis seen in irradiated recipients acutely infected with LCMV is blocked if these recipient mice are treated with antibody to asialo GM1 (THOMSEN et al. 1986). It is thus speculated that irradiation-resistant NK cells inhibit hematopoiesis during acute LCMV infection. Supporting this hypothesis is that mixture of human bone-marrow cells with human PBL in vitro inhibits bone-marrow cell outgrowth, as measured in assays in vitro (HANSSON et al. 1981; DEGLIANTONI et al. 1985). These effector cells have an NK-cell phenotype. Further, marked similarities are noted between the murine NK system and the effector system of hybrid resistance, that is the system which, in irradiated mice, results in the rejection of F1-antiparent bone-marrow grafts (KIESSLING et al. 1977; DALEY and NAKAMURA 1984). Thus, NK cells inhibit CFU outgrowth when mixed with bone-marrow cells in vitro, the peak in hematopoietic dysfunction correlates with the peak in NK-cell activity during acute LCMV infection in vivo, and depletion of the NK activity in vivo in at least one system of study results in greater hematopoietic function.

7.3 Antibody Production

Recent reports indicate that NK cells may inhibit B-cell function and antibody formation (ABRUZZO and ROWLEY 1983; SHAH et al. 1986). One possible mechanism suggested is that NK cells lyse antigen-presenting cells, such as dendritic cells (SHAH et al. 1985). It is possible that the high levels of activated NK cells may inhibit antibody production during acute LCMV infection, and this could be a reason why antibody plays little part in the acute LCMV disease. This is a speculative issue which should be examined experimentally.

7.4 Resistance to Heterologous Viruses

Mice persistently infected with LCMV are resistant to superinfection with a number of viruses (DALFDORF 1939; HOTCHIN and CINITS 1958; TRAUB 1961). This is likely to be at least partially due to chronic low levels of IFN made in those mice (SARON et al. 1982; BUKOWSKI et al. 1983b). However, the slightly augmented NK-cell response associated with the elevated IFN levels may play a role in resistance to NK-sensitive viruses as well (BUKOWSKI et al. 1983b). In this respect, the LCMV-carrier mice, by being more resistant to other viruses

and to tumor formation, may actually maintain an NK-cell–dependent selective advantage over normal mice.

8 Summary and Conclusions

Arenavirus infections, most notably those of LCMV and PV, have provided useful experimental models for examining the properties and functions of cytotoxic lymphocytes in virus infections. It can probably be predicted that CTL are important in the regulation of most if not all of these infections, whereas NK cells regulate only some of the infections. More work is needed and is currently ongoing to determine the contributions of these effector cells in other arenavirus infections and, in particular, in human arenavirus infections. Because the T-cell response to arenavirus infections is critically reviewed elsewhere in this volume, I have deliberately focused this review on NK cells. However, because of the similarities in phenotypes and the dynamics of the NK- and T-cell responses, attempts were made to compare and contrast these responses and to focus on the cytotoxic properties of these cell types.

The cytotoxic lymphocyte response to the LCMV infection can now be summarized as follows. LCMV enters the body and begins to replicate in a variety of cell types, inducing the synthesis of IFN type I. IFN activates and induces the proliferation of NK cells. The virus spreads to different parts of the body, undergoes further replication, induces more IFN, and NK cells accumulate at these sites of infection. The high levels of IFN induced alter the cells of the body biochemically, by enhancing expression of histocompatibility antigens, sialic acid, neutral glycosphingolipids, and gangliosides. Associated with these biochemical changes is a conversion of the cells in the body to a resistant state against NK cells. Both uninfected and LCMV-infected target cells become resistant to NK cells, limiting the potential autodestructive capacities of the NK cells as well as their ability to limit LCMV synthesis. These activated NK cells may, however, contribute to an inhibition of hematopoiesis by lysing certain precursor cells in the bone marrow. While all this is happening, clones of specific CTL are being expanded. Increased H-2 antigen expression on LCMV-infected target cells and antigen-presenting cells may contribute to a further augmentation of the immune response. Lymphokines liberated during this potent immune response activate other cells in the immune system, including macrophages and memory CTL with specificities not cross-reactive with LCMV. The LCMV-specific CTL lyse LCMV-infected target cells, whose class I H-2 antigens have been greatly augmented by IFN. This causes a reduction in virus synthesis, which results in a concomitant reduction of IFN synthesis. After removal of this IFN stimulus, the NK cells stop proliferating and return to a normal endogenous level of activation. In the case of an animal infected i.c. T-cell–dependent immunopathology results in meningoencephalitis and death of the animal.

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Immunotherapy for Virus Infection

M.B.A. OLDSTONE

1 Introduction

1.1 Balance Between Acute and Persistent Infection

Understanding of virus-induced immune response disease and the related immunopathology has its roots in the studies reported by ROWE (1954). He showed that suppression of immune responses changed the ordinarily lethal, acute infection with lymphocytic choriomeningitis virus (LCMV) to a persistent infection in susceptible mice. The subsequent work of many investigators who used neonatal thymectomy, genetically athymic mice, irradiation, antilymphoid drugs, or antithymocyte sera, etc. (reviewed in BUCHMEIER et al. 1980) extended the concept of immune response-mediated injury during viral infection. Thereafter, the role of lymphocytes in mediating virus-induced immunologic injury was delineated. First, experiments performed independently by LUNDSTEDT (1969) and OLDSTONE et al. (1969) showed that lymphocytes obtained 6–9 days after an acute LCMV infection or primary inoculation killed LCMV-infected targets in vitro. Next, such lymphocytes were found to be of thymic origin and to bear Thy 1.2 markers of their surfaces (COLE et al. 1973; MARKER and VOLKERT 1973). GILDEN et al. (1972a, b) showed that mice which survived an ordinarily lethal dose of LCMV owing to immunosuppression developed acute lymphocytic choriomeningitis (LCM) disease and died when reconstituted with syngeneic,

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immune T-lymphocytes. ZINKERNAGEL and DOHERTY (1974a) defined the need for two signals between the cytolytic T-lymphocyte and its infected target, namely, virus specificity and H-2 restriction, so that killing of LCMV-infected cells could proceed. Armed with these data, others (MIMS and BLANDEN 1972; ZINKERNAGEL and WELSH 1976) used virus-specific splenic lymphocytes to reconstitute immunosuppressed, infected H-2–matched recipients and thereby lowered their viral titers.

Naturally occurring persistent LCMV infection was first recorded by TRAUB (1936), who observed retention of infectious virus in tissues and circulation throughout the animals' life spans. Two decades later HOTCHIN and CINTIS (1958) described a model whereby newborn mice, inoculated with LCMV within the first 24 h of life, survived an ordinarily lethal dose of virus (the same dose or one several logs less was lethal to adult mice) and showed continuous infection in sera and tissues over the natural course of life. This model provided a clinical and biological picture through experimental manipulation similar to the naturally occurring disease described by TRAUB. Several investigators have demonstrated that such persistently infected mice are relatively deficient in virus-specific immune lymphocytes, but are able to mount high titers of antiviral antibodies (reviewed in BUCHMEIER et al. 1980; OLDSTONE 1979).

Figure 1 shows the balance between the acute and persistent LCMV infection and the role of immune factors during the course of both infections. Inoculation of immunocompetent adult mice with LCMV by the intracerebral route usually leads to acute leptomeningitis, choroiditis, and inflammation of the ventricle, leading to death within 6–10 days. Cumulative evidence indicates that cytotoxic T-lymphocytes (CTL) are the major effector cells in this reaction (see ALLAN, DIXON, and DOHERTY, this volume). When virus is introduced into adult immunocompetent animals by the peripheral route, animals may either survive or die dependent on the balance struck favoring either immunity or immunopathologic injury. When newborn mice, less than 24 h of age, are inoculated by any route, including intracerebral, virus persists in tissues and sera throughout the animals' lives. As shown below, in Sect. 3.2, reconstitution with immune lymphocytes leads to clearance of viral materials. When adult immunocompetent mice are inoculated with an ordinarily lethal dose of virus coupled with immunosuppression given as irradiation, thymectomy, antilymphocyte serum, or cytoxan, a persistent infection occurs. In comparison with persistent infection established through inoculation of newborn animals, the sites of viral antigen in the adults are primarily the leptomeninges and lining of the ventricles. Transfer of LCMV-primed heterogeneous populations of lymphocytes or cloned CTL causes immunopathologic disease (BAENZIGER et al. 1986; BYRNE and OLDSTONE 1986). However, following infection as newborns, persistently infected adults express virus materials primarily in neuronal cells of the central nervous system (OLDSTONE and DIXON 1969; RODRIGUEZ et al. 1983). Similarly, when adult immunocompetent mice are inoculated intravenously with a lymphotropic-derived LCMV variant that suppresses CTL activity, virus persists in multiple sites including the neuronal cells (AHMED et al. 1984; TISHON and OLDSTONE 1986). However, the virus then replicates primarily in the nervous system, with limited expression in cells lining the ventricles. Transfer of immune lymphocytes

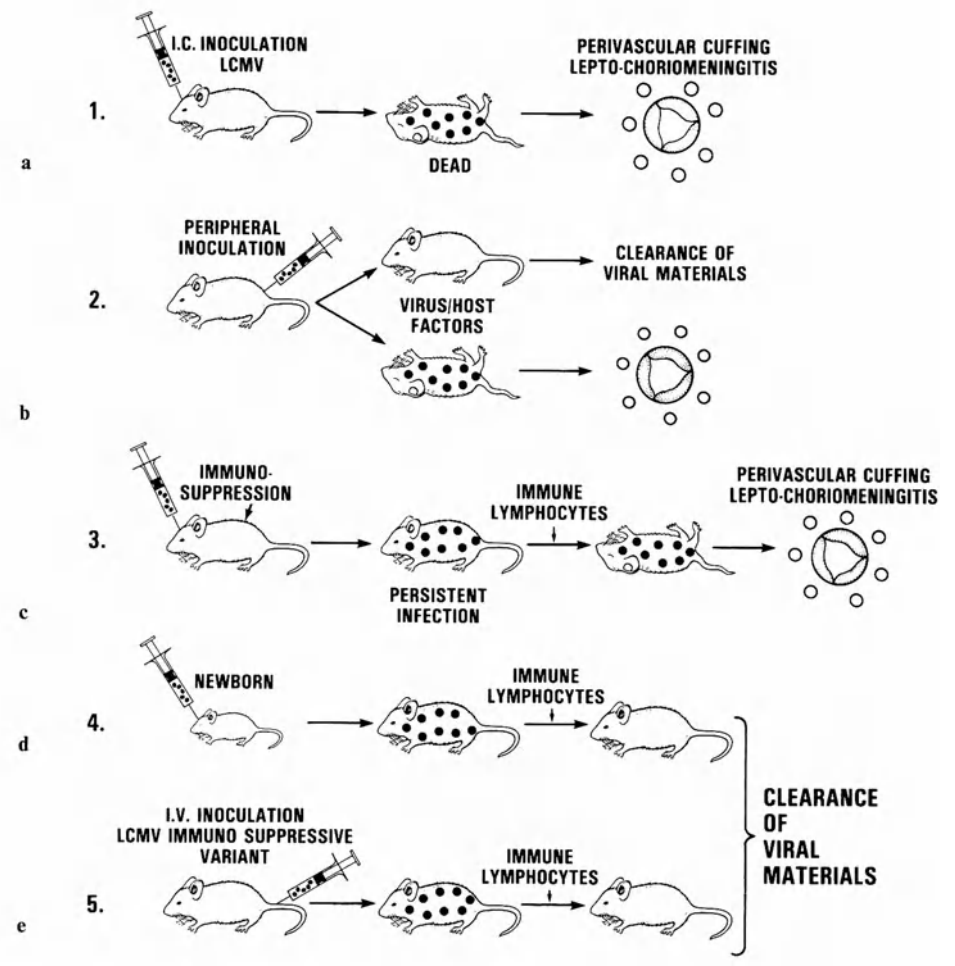


Fig. 1 a–e. Experimental model system for establishing acute and persistent LCMV infection. **a** Intracerebral inoculation of adult immunocompetent animals with significant amounts of wild-type LCMV strains leads to death within 6–10 days associated with perivascular cuffing and infiltration into the lymphomeninges, choroid plexus, and ventricles. **b** Peripheral inoculation of adult immunocompetent animals dependent on the route of inoculation and dose, the genetic background of the host, and other factors leads either to clearance of viral materials and immunity or death with a histopathologic picture similar to that of mice dying after intracerebral inoculation. **c** Adult immunocompetent mice inoculated intracerebrally with an ordinarily lethal dose of virus and receiving immunosuppression by a variety of means (irradiation, thymectomy, antithymocytic or lymphocytic sera, cytoxan, etc.) develop a persistent infection. Virus is found primarily in the leptomeninges and ependymal cells lining the ventricles (GILDEN et al. 1972a, b). Adoptive transfer of LCMV-immune H-2 restricted lymphocytes rapidly causes death, much like the intracerebral injection of adult immunocompetent animals did. **d** In contrast, inoculation of newborn mice (<24 h old) with most wild-type strains of LCMV or **e** inoculation of adult immunocompetent mice with LCMV immunosuppressive variants (AHMED et al. 1984; TISHON and OLDSTONE 1986) leads to persistent infection with virus expressed in most tissues of the body and found primarily in neurons of the central nervous system. Transfer of immune lymphocytes that are virus specific and H-2 restricted promotes clearance of the viral materials

to such neonatally infected or adult immunosuppressed animals precludes the development of acute LCMV disease; instead viral materials are cleared (OLDSTONE et al. 1986).

1.2 Generation of Immune Reactants: Antibodies, CTL, NK Cells

Following the initiation of infection and the replication of virus at the site of initial introduction, infection spreads to distant sites. Inoculation intracerebrally is similar in many respects to that done intravenously as virus in the brain soon ruptures the ventricle and rapidly enters the circulation. Several immune reactants are made by the host exposed to virus, the primary functions of which are to limit the spread of viral infection and/or remove virus-infected cells. The removal of virus-infected cells eliminates factories where infectious materials are made and can be achieved by either antiviral antibodies or cytotoxic lymphocytes, preferably before the release of progeny virus (ZINKERNAGEL and ALTHAGE 1977; ANDERSON et al. 1985). A wide spectrum of host responses is possible, and each plays a greater or lesser role depending on the time of induction, the site where virus is found, and the characteristics of that particular virus infection.

As shown in Fig. 2, for most strains of immunocompetent mice studied, intraperitoneal injection of 10^2 to 10^5 plaque-forming units (PFU) of LCMV

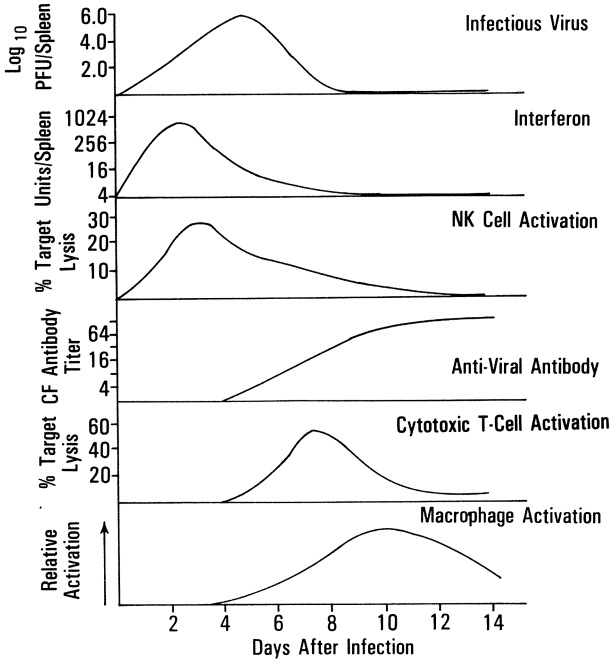


Fig. 2. A cartoon that combines experimental data from many investigators and laboratories and shows the host response to acute LCMV infection following intraperitoneal inoculation (see BUCHMEIER et al. 1980 for details). CF, complement fixing antibody; NK, natural killer

results in the following: viremia peaks at about the 4th–5th days postinfection and then rapidly declines. Little if any infectivity is detected in the blood beyond the 8th–9th day postinfection. Interferon is measurable by the 1st day postinfection and reaches maximal titers around the 3rd day, then continuously decreases to baseline levels by the 8th–9th day. Concurrent with interferon synthesis is the generation of activated natural killer (NK) cells, which may be directly activated by interferon (WELSH 1978). Studies in which NK cells were depleted from LCMV-infected mice in vivo (see WELSH, this volume) indicate that such cells play a relatively minor role. Also at the time of peak interferon synthesis a marked reduction in hemopoietic function occurs, which may not return to normal until the 2rd week postinfection (BRO-JORGENSEN 1978; R.E. KIESSLING, personal communication. LCMV-specific H-2–restricted CTLs begin to appear on the 5th day and peak on the 7th–9th days postinfection. As shown in Sect. 2.2, CTLs play an important role in the clearance of virus materials from infected animals. Antibodies to virus are present as early as the 3rd–4th day postinfection, and on the 5th day one finds highly activated macrophages. The roles of antibody in neutralizing arenaviruses, and of large granular lymphocytes and T-lymphocytes in clearing virus and mediating delayed type hypersensitivity are covered in HOWARD, MARKER and RANDEUP, THOMSEN, and WELSH (this volume), while the immunopathology associated with LCMV acute infection is described in ALLAN et al. (this volume). This chapter is devoted to the clearance of viral materials from acute and persistently infected mice by virus-specific class I MHC-restricted lymphocytes.

2 Acute LCMV Infection

2.1 Historical Background

Studies with LCMV-infected mice provided the first reports that virus-specific CTLs existed both in vitro (COLE et al. 1973; MARKER and VOLKERT 1973) and in vivo (GILDEN et al. 1972a, b). These observations that CTLs killed virus-infected cells were confirmed by several groups for LCMV infection and then extended to other RNA and DNA viruses known to infect man and animals (reviewed by ZINKERNAGEL and DOHERTY 1979). Detailed analysis of the host genetic background required for CTL activity led ZINKERNAGEL and DOHERTY to define CTL killing on the basis not only of a Thy 1 marker and the ability to kill specific virus-infected targets, but also on the absolute requirement for H-2 compatibility (K and/or D end of the major histocompatibility complex, MHC) between the CTL and the infected target (ZINKERNAGEL and DOHERTY 1974). Thereafter, independently made observations documented that appropriate experimental manipulations involving adoptive transfer of cytotoxic lymphocytes could clear viral infection in vivo (MIMS and BLANDEN 1972; ZINKERNAGEL and WELSH 1976). In both instances, supplementing acutely LCMV-infected mice with heterogeneous populations of LCMV-primed spleen cells that contained CTLs reduced the titer of virus. However, because such transfers included

primed T helper cells, B cells, null, and NK cells, and macrophages, a precise definition of this phenomenon awaited the development and use of cloned CTLs.

2.2 Clearance of Viral Materials from Infected Animals with Cloned, Virus-Specific, H-2 Restricted CTLs

LCMV-specific, H-2 restricted CTL clones were generated from spleens of mice immunized 30–90 days earlier with LCMV (BYRNE et al. 1984). Table 1 lists several of the CTL clones developed, their H-2 genotypes, their efficiency of killing LCMV-infected targets at low effector to target (E:T) ratios within a 5-h ^{51}chromium release assay, their virus specificity (i.e., their inability to kill cells infected with Pichinde virus – another member of the arenavirus group that does not cross-react with LCMV at the glycoprotein level), and their H-2 restriction. Such cloned CTLs, by themselves, in in vitro experiments, prevented the release of infectious virus into culture fluids by destroying the factories of virus production and thereby significantly lowering the titers of infectious virus compared with cultures not incubated with CTL (ANDERSON et al. 1985). Thus, CTLs in the absence of T-cell help, macrophages, NK cells, or antibodies can reduce virus titers. The ability of cloned CTLs is not influenced by monensin, a compound believed to inhibit the exit of membrane glycoproteins from the Golgi complex (TARKAROFF 1983). Therefore, lysis by committed cloned CTLs apparently does not require newly processed glycoproteins. If such glycoproteins are involved in a lytic event, they may already be present in the membrane or at the cytoplasmic sites in membrane-bound inclusions. In contrast, noncloned CTLs (virus-specific and H-2 restricted) obtained from spleens of infected mice are susceptible to the effects of monensin. Thus, monensin is a useful material for segregating the activity of these two CTL populations; comparison

Table 1. Generation of LCMV-specific, H-2 restricted CTL clones (see BYRNE et al. 1984 for details)

CTL Clone		Specific ^{51}Cr release (E:T 2.5:1) (%)				
No.	H-2	LCMV-Specific			H-2 Restricted	
		LCMV	Pichinde	Uninfected	H-2b	H-2d
b-9-1	K^bD^b	96	0	2	96	0
b-11-2	K^bD^b	93	1	2	93	1
b-11-5	K^bD^b	93	2	3	93	0
b-11-13	K^bD^b	64	1	4	64	0
d-5-60	K^dD^d	99	3	3	4	99
85-d-1	K^dD^d	73	8	0	0	73
85-d-3	K^dD^d	65	0	1	0	65
85-d-4	K^dD^d	84	12	0	1	84
85-d-6	K^dD^d	73	3	0	0	73

suggests that cloned CTLs may be at a later stage of differentiation than primed splenic CTLs used in the early adoptive transfer experiments (MIMS and BLANDEN 1972; ZINKERNAGEL and WELSH 1976). When tested for the participation of lymphokines, the CTL clones proliferated after reacting with syngeneic LCMV-infected macrophages, but failed to make interleukin-2. The cloned

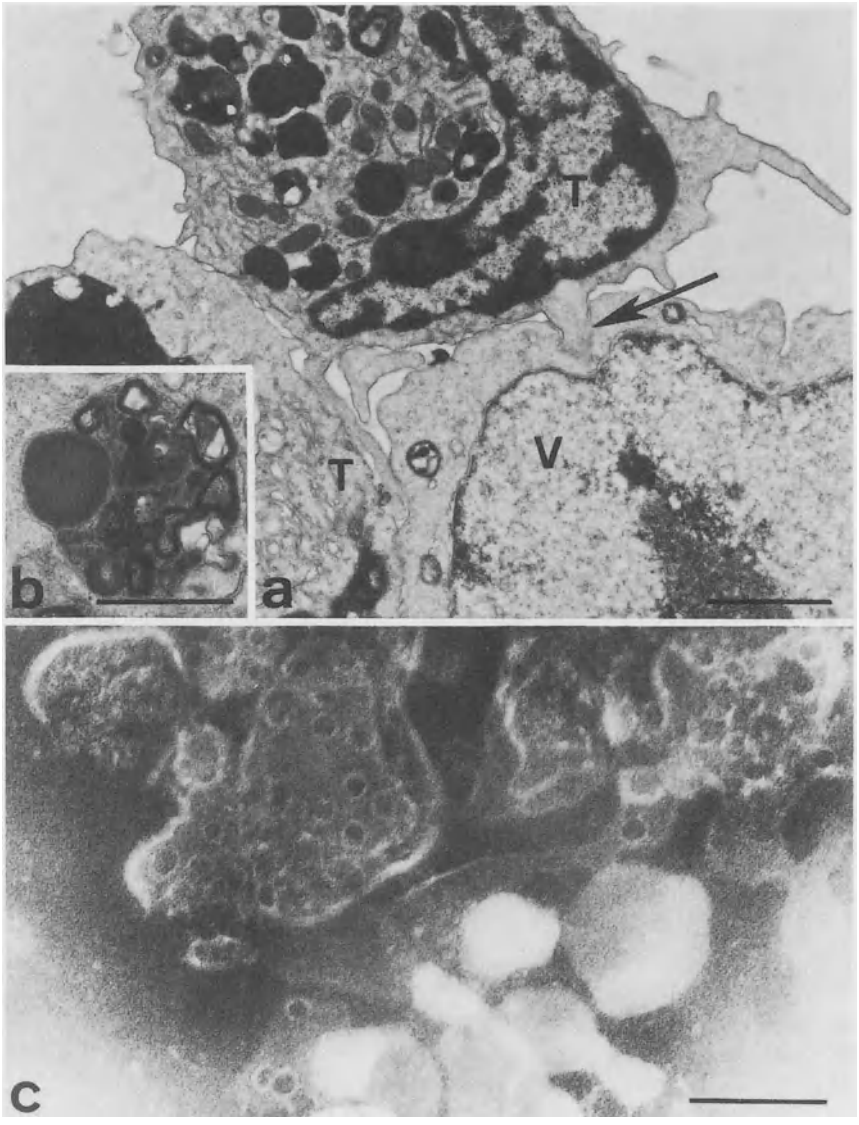
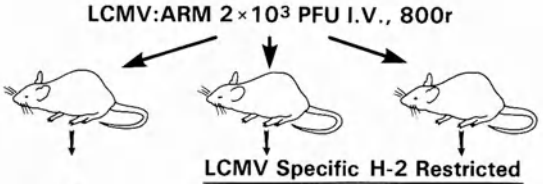


Fig. 3a–c. Interaction between a cloned LCMV-specific, H-2 restricted CTL and LCMV-infected target cell. **a** Two CTLs (*T*) bind to an LCMV-infected target cell (*V*). A penetrating CTL process (*arrow*) almost reaches the nuclear membrane of the target cell. The *bar* represents 2 μm. **b** CTL membrane-bound cytoplasmic inclusion containing an electron-dense granule and multilaminar membrane structures. The *bar* represents 1 μm. **c** Lesions on the membrane of a cell lysed by CTLs. The *bar* represents 100 μm. (See ANDERSON et al. 1985 for details)



		LCMV Specific H-2 Restricted	
Day 1	NIL	CTL Clone	Day 7 P° CTL
	PFU	PFU	PFU
Day 2	SPL LN	SPL LN	SPL LN
	6.0 3.0	<1.7 2.8	<1.7 <1.7
	5.6 2.8	2.5 2.8	<1.7 <1.7
	5.6 2.8	<1.7 3.0	<1.7 <1.7
Day 5	4.3 3.5	<1.7 4.3	<1.7 <1.7
	4.5 3.6	<1.7 3.6	<1.7 <1.7
	4.3 4.2	<1.7 2.9	<1.7 <1.7
	4.3 3.0	<1.7 2.8	<1.7 <1.7

Fig. 4. Experimental protocol demonstrating the ability of LCMV Armstrong strain (*ARM*)-specific, H-2 restricted CTL clones to clear virus from splenic but not lymph node tissues. One day after introduction of virus, CTL clones were administered, and 1 or 4 days later (day 2 and day 5 of infection, respectively) tissues were removed and assayed for infectious virus by plaque assay. Both the replication of LCMV in mice not receiving immunotherapy (*NIL*) and in mice receiving a primed heterogeneous population of virus-specific, H-2 restricted splenocytes 7 days after viral inoculation (*day 7 P^0 CTL*) are shown (see BYRNE and OLDSTONE 1984, 1986 for details)

CTLs made gamma interferon when reacted with syngeneic, virus-infected targets. However, the production of interferon did not directly correlate with CTL mediated killing and, in agreement with these findings, the numbers of H-2K and D molecules expressed on the surfaces of virus-infected target cells was not altered during the course of LCMV infection (ANDERSON et al. 1985). Electron microscopy showed fingerlike projections of the CTL clones thrust into infected cells in vitro and lesions bearing an internal diameter of approximately 15 nm in those membranes undergoing the lytic process (Fig. 3).

Adoptive transfer experiments with cloned CTLs indicated that T-lymphocytes with virus-specific, H-2 restricted cytotoxic activity in vitro could clear virus infection and maintain clearance for over 5 days in splenic tissue, but not in mesenteric lymph nodes (BYRNE and OLDSTONE 1984, 1986). Figure 4 shows studies of several individual mice at 2 and 5 days after adoptive transfers of 1×10^7 cloned CTLs. Other experiments indicated that as little as 3×10^6 cloned cells were effective in reducing virus titers. The clearance of infectious virus ranged from 3–5 logs; this activity was genetically restricted to class I MHC antigens and was virus specific in that similar adoptive transfer did not reduce titers of mice infected with Pichinde virus. Additionally, the migration of CTLs in vivo was virus specific and H-2 restricted (BYRNE and OLDSTONE 1986).

Reassortants of LCMV were then used to map CTL recognition to gene products (G1; G2; nucleoprotein, NP) encoded by the viral S RNA segment (RIVIERE et al. 1986). Recently, WHITTON et al. (1986) inserted a cloned, truncated LCMV Armstrong gene containing 262 amino acids of G1 and 100 amino acids of G2 into a vaccinia vector, and showed that these LCMV glycoproteins were sufficient to allow H-2 restricted, LCMV-specific CTL recognition in vitro.

3 Persistent LCMV Infection

3.1 Historical Background

Figure 1 records several of the principles of immune clearance observed in mice persistently infected with LCMV. The mechanism(s) by which LCMV is cleared in vivo during acute infection is now relatively well-understood (see above). Clearance, although associated with several antiviral immune effector mechanisms (Fig. 2), is primarily dependent on the activity of virus-specific, H-2 restricted CTLs (COLE et al. 1972; ZINKERNAGEL and DOHERTY 1974; ZINKERNAGEL and WELSH 1976; BYRNE and OLDSTONE 1984). Failure to generate virus-specific CTLs or depletion of such cells allows progression from acute to persistent infection. In the latter situation, infection persists in many organs with infectious virus reaching its highest titers during the initial days after injection, approximately 10^7–10^8 PFU/ml of serum or g of tissue, and dropping 4–5 logs

Table 2. Results of an in vitro CTL assay using virus-specific (LCMV), nonvirus-specific (Pichinde), and H-Z restricted (H-2d) and unrestricted (H-2b) target levels. LCMV-specific, H-2 restricted CTL killing is selectively diminished during persistent LCMV infection. Pichinde virus is an LCMV-related arenavirus that does not cross-react at the CTL level with LCMV. With a 5-h ^{51}chromium-release assay, the inability of persistently, infected mice to generate CTLs spontaneously or after inoculation with LCMV (not shown) is recorded. However, such mice persistently infected with LCMV are able to generate a Pichinde virus CTL response

Status of H-2d BALB/W mouse		Percent specific ^{51}Cr release				
		Virus infected H-2d				LCMV H-2b
	No.	LCMV		Pichinde	Uninfected	
		50:1	5:1	50:1	50:1	50:1
Mock infected	1	8	2	8	5	4
	2	3	0	9	7	2
	3	5	3	2	6	3
	4	6	2	5	1	5
Acute LCMV infection (day 7 P° SPL)	1	81	28	7	6	6
	2	76	26	8	4	7
	3	76	12	5	3	4
	4	61	18	8	0	1
Persistent LCMV infection	1	6	3	4	5	2
	2	16	5	9	0	4
	3	8	4	3	1	6
	4	12	5	4	2	0
Persistent LCMV infection (Pichinde day 7 P° SPL)	1	8	3	54	4	4
	2	7	2	63	3	2
	3	14	5	58	5	1
	4	12	4	46	1	1

over the next several days until it reaches 10^3–10^5 PFU/ml, where it remains for life. In contrast, viral nucleic acid sequences are barely detectable in tissues during the initial phase of heightened viral replication, but steadily accumulate thereafter over the course of infection (SOUTHERN et al. 1984; FRANCIS et al. 1986) suggesting that incomplete or defective viruses are formed in vivo. During persistence, antibodies to all LCMV proteins are made, but the virus-specific, H-2 restricted CTL response is decreased (Table 2), a selective decrease that suggests at least one specific defect enabling persistent infection to endure.

These results suggest that mice persistently infected with LCMV then reconstituted with LCMV-specific CTLs might clear viral nucleic acid sequences, viral proteins, and infectious virus. Initial studies to test this hypothesis began 2 decades ago (VOLKERT and HANNOVER LARSEN 1964, 1965). Although the transferred lymphocytes cleared virus from the blood and several tissues, the authors did not address the clearance of viral nucleic acids, known to persist and accumulate in such mice nor, at the time, were reagents available for determining the lymphoid cell responsible for clearance. Nevertheless, these studies along with earlier studies reported by GILDEN et al. (1972) formed the foundation for much of our current work.

3.2 Clearance of Viral Materials

The protocol used to determine the clearance of infectious virus (PFU), clearance of viral nucleic acid sequences (whole animal in situ hybridization; see SOUTHERN et al. 1984 and BLOUNT et al. 1986 for details), and decreased expression of viral proteins – immunofluorescence and whole animal in situ hybridization with monoclonal antibodies or antibodies to predetermined sequences and [^{125}I]staphylococcal protein A (see BLOUNT et al. 1986 and FRANCIS et al. 1986 for details) – is shown in Fig. 5. The transfer of either $>3 \times 10^6$ cloned LCMV-specific CTL or splenic lymphocytes from syngeneic mice primed 30–60 days previously with LCMV (memory, immune lymphocytes) and stimulated in vitro with LCMV-infected cells expressing class I and class II MHC antigens into persistently infected mice caused acute immmunopathologic injury leading to death (BYRNE and OLDSTONE 1986). These CTLs, after their introduction intravenously via the tail vein, interacted with viral antigens expressed primarily in the lung and caused immunopathologic injury. For the studies reported below, all adoptive transfers were performed with day 30–60 freshly obtained immune lymphocytes.

Mice persistently infected with LCMV and receiving lymphocytes primed against LCMV cleared both viral nucleic acid sequences and infectious virus. Figures 6 and 7 show the clearance of viral nucleic acids and viral proteins from adult mice whose persistent infection was initiated at birth. Results were similar in adult immunocompetent animals whose persistent infection was initiated in adulthood with the lymphotropic LCMV variant clone 13 (AHMED et al. 1984). Figure 8 shows the clearance of viral proteins from adult mice whose infection was initiated congenitally. Figure 6 also addresses the issues of virus specificity, H-2 restriction, and kinetics of clearance. As shown in the upper

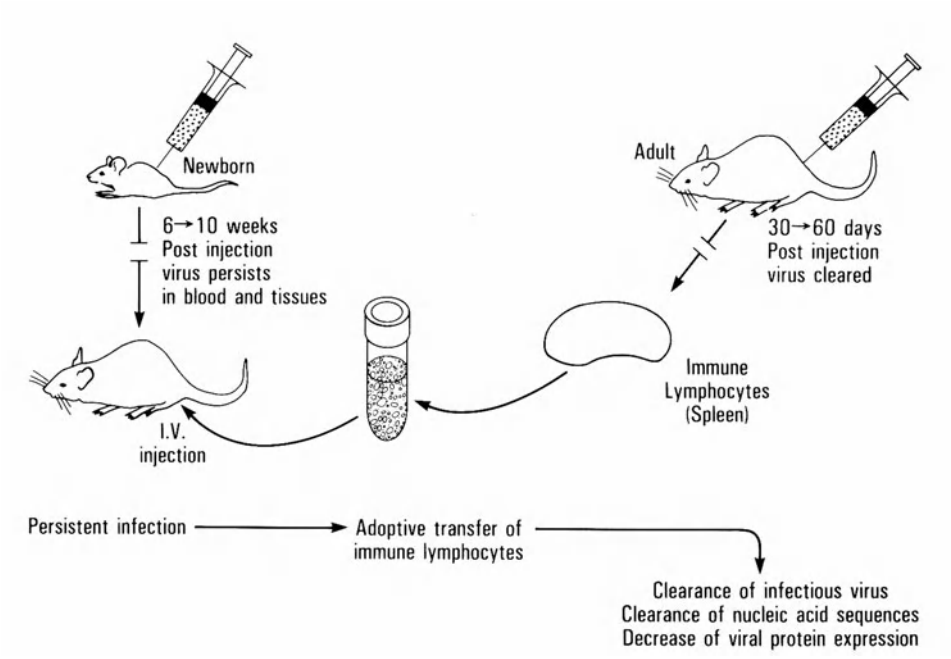


Fig. 5. The protocol to be followed for measuring clearance of infectious virus (PFU), clearance of viral nucleic acid sequences (cDNA probe and in situ hybridization), and viral protein expression (antiviral antibodies, [^{125}I] Staphylococcal protein A and in situ hybridization, or fluorescent antibody detection)

and middle five panels, viral nucleic acid material is cleared only from mice receiving immune splenocytes that are H-2 restricted and virus specific. Hence, properly H-2 restricted but not virus-specific (directed against Pichinde) splenocytes failed to clear viral nucleic acid sequences, whereas LCMV-specific but not H-2 restricted CTLs failed to clear virus from either H-2d BALB (B) or H-2q SWR/J (S) persistently infected mice. Similarly, neither viral nucleic acids nor infectious virus were cleared by H-2 restricted but nonimmune lymphocytes. The bottom five panels in Fig. 6 show a unique pattern in clearance of viral materials from the kidney and brain. Although viral materials were cleared from most organs (liver, lungs, and spleen) 15 days after adoptive transfer of virus-specific H-2 restricted lymphocytes, there was no corresponding clearance in either the central nervous system or kidneys. The majority of viral nucleic acid sequences remained in the kidney and nervous system tissue 30 days after transfer, and not until 120 days had passed was clearance complete in the nervous system. These results were seen in repeated experiments with several different strains of mice and showed that, whereas 75% (over 60 mice studied) of mice cleared virus materials from most organs within 15 days, $<19\%$ showed clearance by 30 days in their brains or kidneys.

Figure 7 complements these studies by demonstrating the remarkable clearance of viral proteins from livers and spleens of mice adoptively transferred

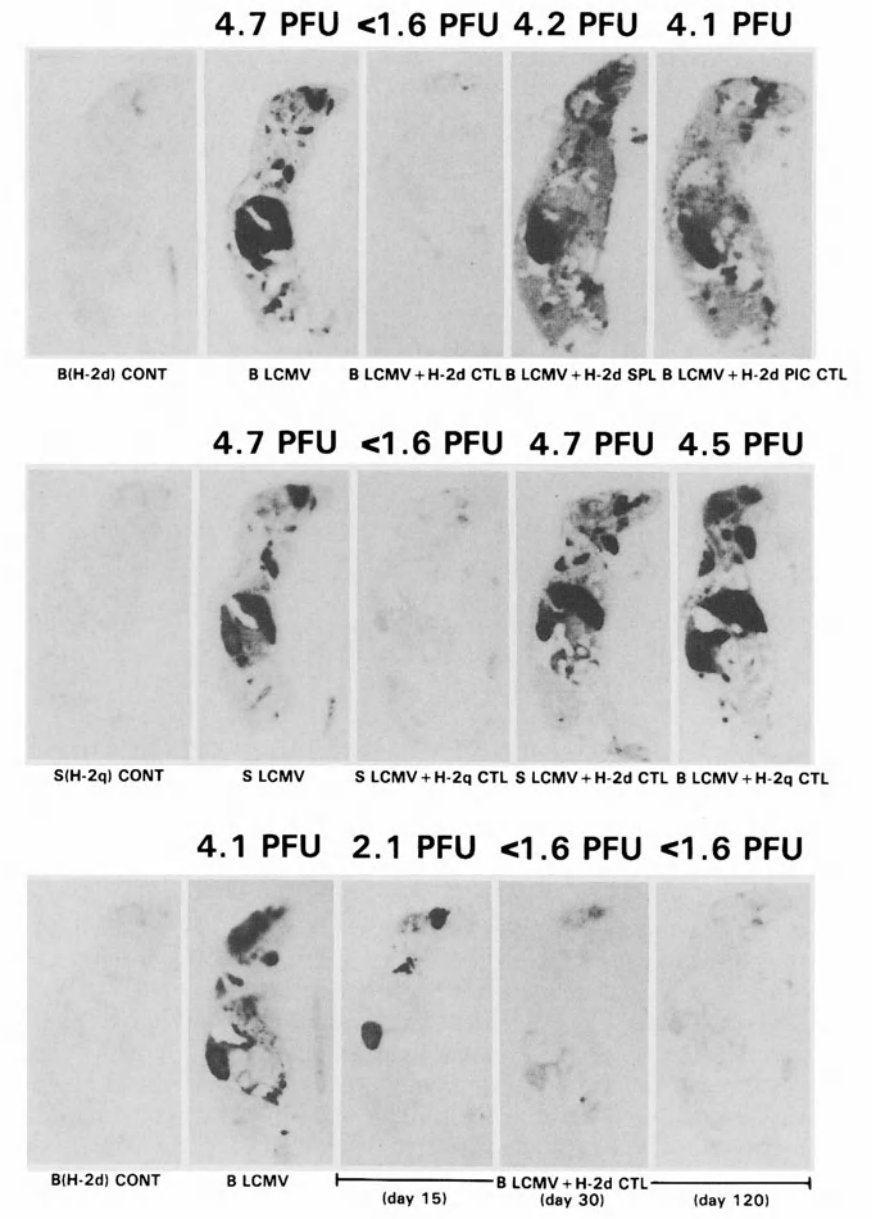


Fig. 6. Immunotherapy clears viral nucleic acid sequences and infectious virus from persistently infected mice. Clearance follows adoptive transfer of lymphocytes that must be specifically primed against the persisting virus and H-2 restricted with respect to the infected host. Clearance differs temporally with a longer period required to remove viral nucleic acid sequences from the brain and kidney than other tissues. Mice were killed when 4–6-months-old and 120 days after adoptive transfer of lymphocytes, except those represented in the *lower panel* as marked 15 and 30 days after adoptive transfer. Cells transferred were splenic lymphocytes (5×10^7 cells) harvested from mice primed (1×10^4 PFU) 30–60 days earlier with either LCMV or Pichinde (*PIC*) arenaviruses. The mice used were BALB/WEHI (B) H-2d or SWR/J (S) H-2q. All mice were treated identically

with virus-specific, H-2 restricted immune lymphocytes. In addition, Fig. 7 shows changes in the distribution of viral antigen patterns in the kidney 60 days after adoptive immune therapy. Analysis of kidneys using autoradiography and fluorescence microscopy indicates the marked accumulation of virus-antibody immune complexes following immunotherapy. Similarly, the notable clear-

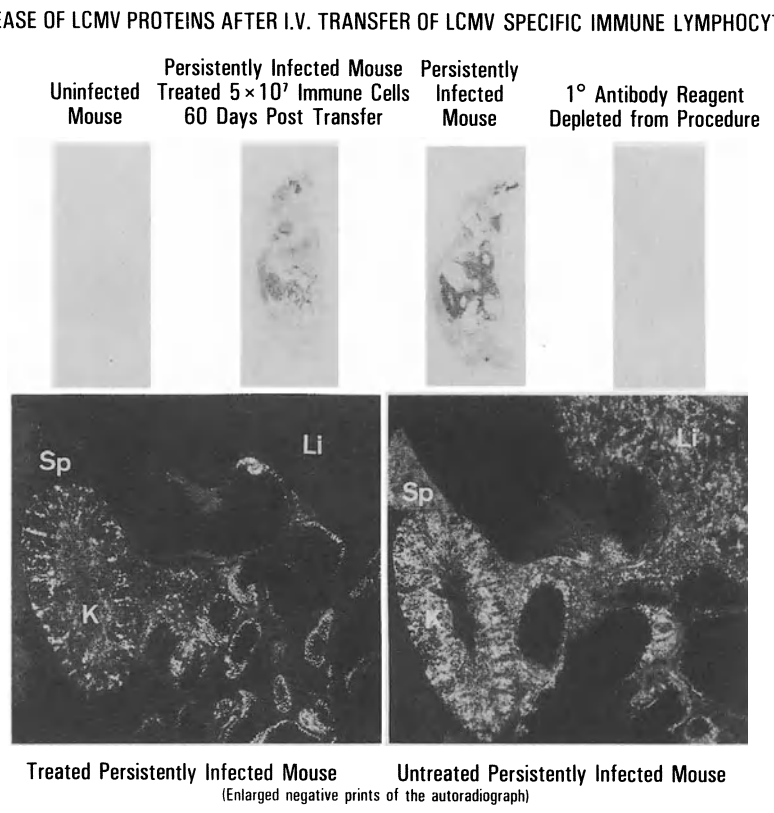


Fig. 7. Clearance of viral proteins from the liver and spleen 60 days after adoptive transfer of immune cells. The *upper panels* show the protein blotting technique on whole mouse sections with appropriate controls (see BLOUNT et al. 1986 for details). The *lower panel* shows an enlargement of the abdomen of persistently infected mice both treated and untreated with immune splenocytes. Note the clearance of viral proteins from the liver (*Li*) and spleen (*Sp*). Also note the change of distribution of viral proteins in the kidney (*K*) presumably being trapped in glomeruli as immune complexes

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and prepared for nucleic acid hybridization using a 545 bp, ^{32}P, nick-translated cDNA probe specific for the 5′ S RNA of LCMV: ARM CA1371 strain (SOUTHERN et al. 1984; OLDSTONE et al. 1986). Infectious virus was titered (PFU) in mouse serum by plaque assay on Vero cells. Values indicate $\log^{10}$ PFU/ml. Negative controls were uninfected BALB/WEHI mice in the *upper row* and *lower row, first panels,* and uninfected SWR/J mice in the *middle row, first panel.* Positive controls consisted of mice persistently infected as newborns by intracerebral inoculation with 60 PFU of virus. Positive controls for BALB/WEHI mice are shown in the *upper* and *lower figures* in the *second panel* and for SWR/J mice in the *middle row, second panel. CTL,* LCMV-specific immune splenocytes from mice primed 30–60 days previously; *SPL,* nonimmune splenocytes

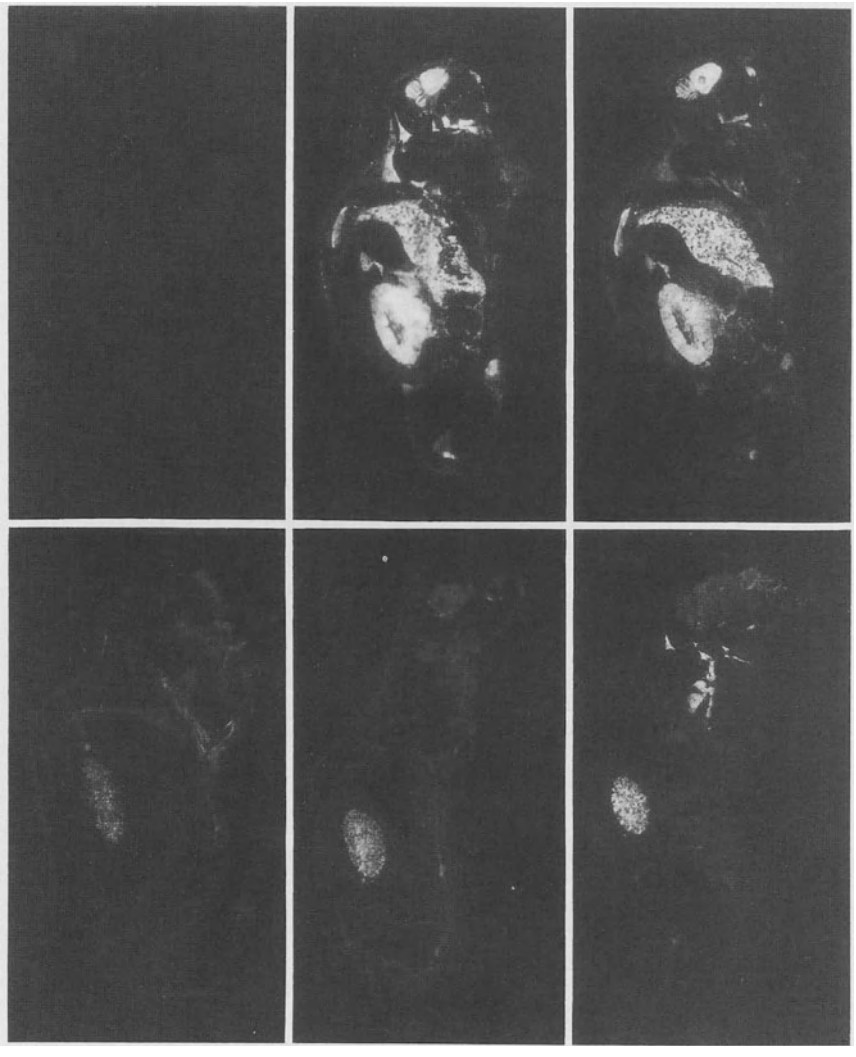

Fig. 8. Clearance of viral materials from C3H/St congenitally infected mice. C3H mice were from the seventh generation of a colony maintained by breeding persistently infected parents. When 6–8 weeks of age, such congenitally infected mice received 5×10^7 memory immune splenocytes intravenously and were killed 15 days after adoptive transfer. The *upper row, first panel,* shows an uninfected age-matched C3H/St mouse probed with antibody to LCMV and [^{125}I]staphylococcal protein A. The *remaining two panels* in the *upper row* show two individual congenitally infected mice that did not receive virus-specific, H-2 restricted immune lymphocytes. The *bottom row* illustrates three individual C3H/St mice receiving immune splenocytes. Note the clearance of LCMV proteins from liver and spleen, the redistribution of viral antigens into an immune complexlike pattern in the renal glomerulus, and the inability to totally clear viral proteins from the central nervous system during this time span

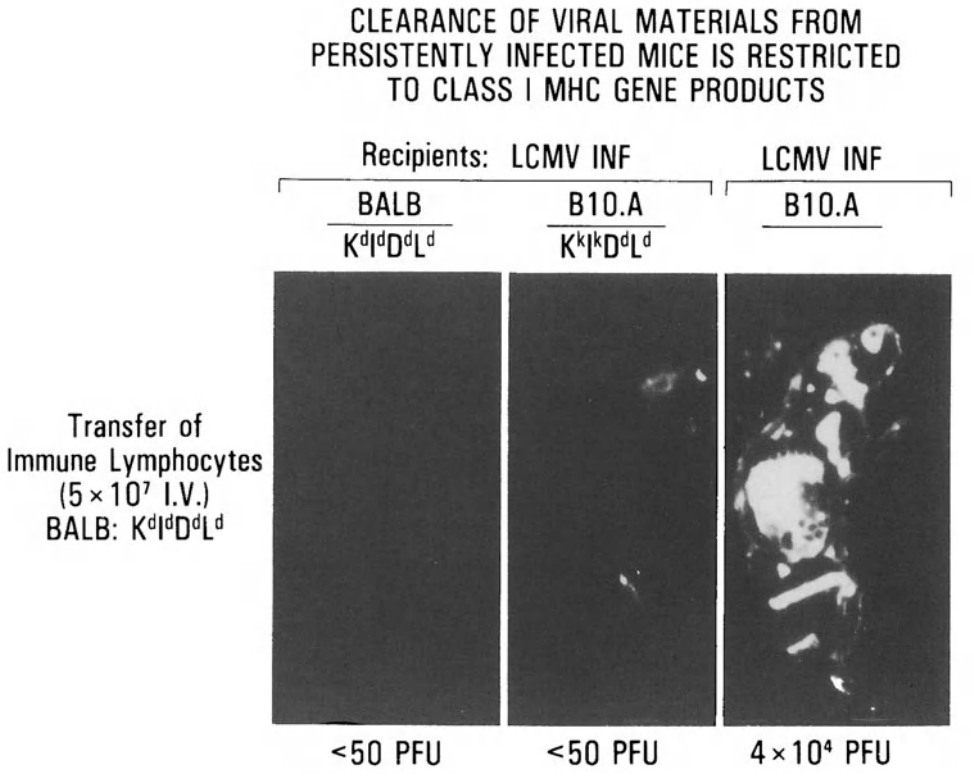

Fig. 9. The H-2 restriction requirement for clearance of viral nucleic acid sequences is restricted primarily to class I *MHC* genes at the D^d, L^d locus. Mice killed 30 days after adoptive transfer with immune lymphocytes

ance of viral proteins from the liver and spleen but not the brain 15 days after adoptive transfer, and the change in antigen presentation, are shown in congenitally infected C3H mice also given immunotherapy (Fig. 9).

Several studies have dissected the H-2 restriction and phenotype of immune lymphocytes involved with the clearing of viral nucleic acid sequences and proteins from persistently infected mice. When LCMV-specific memory splenic lymphocytes obtained from BALB/WEHI mice (H-2K^d, I^d, D^d, L^d) were transferred either into BALB/WEHI (H-2K^d, I^d, D^d, L^d), B10.A (H-2K^k, I^k, D^d, L^d), or SWR/J (H-2K^q, I^q, D^q, L^q) persistently infected mice, a 2- to 4-log decrease in infectious virus from that usually carried in the serum and a decrease in viral nucleic acid materials were observed in B10.A and BALB/WEHI mice, but not in SWR/J mice. These experiments suggest that clearance of viral materials is restricted primarily to class I *MHC* genes at the D^d, L^d locus.

The phenotype of the effector lymphocyte responsible for clearance has also been determined. Elimination of lymphocytes bearing Thy 1.2 or Lyt 2.2 markers from cells used for adoptive transfer prevented the clearance of viral materials. This was accomplished by using the two monoclonal antibodies AD4.15 or YTS169.4. In contrast, depletion of LT34-bearing lymphocytes with

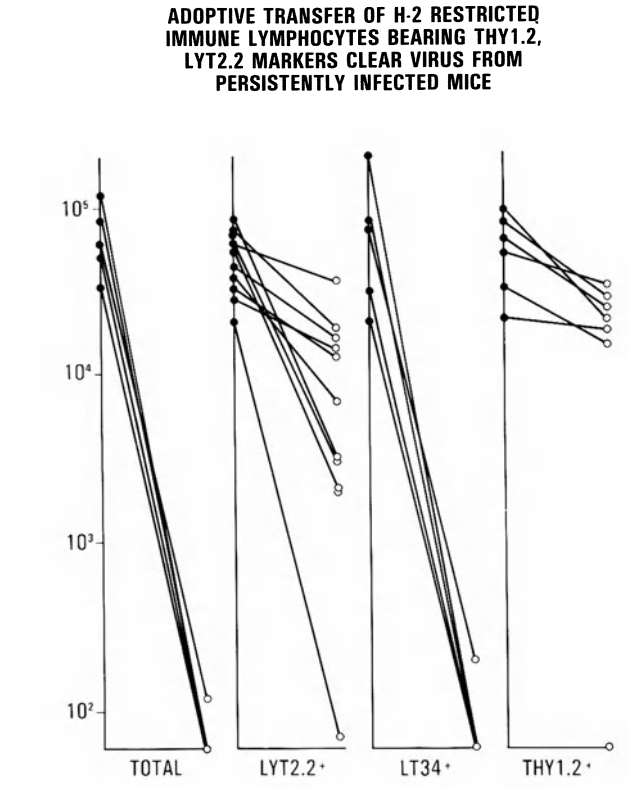

Fig. 10. The phenotypes of immune lymphocytes causing clearance of virus materials from persistently infected animals are Thy 1.2^+, Lyt 2.2^+, and $LT34^-$. Results are from one of several experiments utilizing individual mice. Mice were bled on the day of and prior to immunotherapy (●) and 15 days later (○). Total refers to unfractionated immune splenocytes used for the adoptive transfer. (For experimental details see OLDSTONE et al. 1986)

the monoclonal antibodies GK1.5 and YTS191.1 most often had minimal effects on clearance. Thus, in repeated experiments with three to four mice per group, deletion of the Lyt 2.2 subset did not lower infectious virus titers in the sera prior to immunotherapy by more than a log or a log and a half; in contrast, deletion of the LT34-positive population lowered the virus titer by 2–4 logs. Representative results are displayed in Fig. 10.

4 Conclusion

How infectious materials are cleared by a host has been an area of intensive study and interest. By using molecular probes to record viral nucleic acid sequences and viral proteins and a plaquing assay to detect infectious materials, viruses were found to be efficiently and effectively cleared by immunotherapy utilizing cloned CTLs. Cloned CTLs cleared nearly 4 logs of virus from the spleens of acutely infected animals and, in persistently infected mice, adoptive transfer of virus-specific immune H-2 restricted lymphocytes bearing the Thy 1, Lyt 2^+, and LT34 nil phenotypes cleared viral materials from a wide variety of tissues and organs where they normally lodge. In the acute infection infectious

virus was cleared from the spleens, but not from the lymph nodes, an event that may represent faulty alteration in the migration of the cloned CTLs in vivo or some as yet unexplained event. The efficient clearance of viral materials from persistently infected mice was observed in most tissues but, unexpectedly, removal from the central nervous system differed markedly from the pattern of clearance at other sites. Clearance from the central nervous system was not associated with immunopathologic unjury. This was likely due to the fact that LCMV material settles in neuronal cells, which express limited or negligible amounts of class I and class II MHC products, materials that are necessary for CTL recognition and lysis (WONG et al. 1985; MAIN et al. 1985; ZINKERNAGEL and OLDSTONE 1976; OLDSTONE et al. 1986). Hence, the clearance of viral materials from the central nervous system (neurons) occurs in the absence of the usually detected CTLs or the lysis of infected cells they induce (OLDSTONE et al. 1986; OLDSTONE, unpublished observations). How viral materials are cleared in the absence of cell lysis is unknown, but this act is reminiscent of observations in vitro when interferon or similar lymphokines are present.

One should note that the principles of immunotherapy described here may be effective for the treatment of other persistent microbial infections or malignancies that involve a selective lymphocyte defect. The observation that virus is cleared from neurons without their destruction suggests that this treatment may be useful for many infections of the central nervous system. The recent reports of numerous RNA and DNA viruses that are tropic for lymphocytes and their subsets leading to abrogation or modulation of such lymphocyte functions as cytotoxicity or production of antibodies raise the possibility that reconstitution of these cells may be a valid treatment for both acute and persistent infections.

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Pathophysiology and Treatment of Lassa Fever

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1 Lassa Fever in Man

Infection with Lassa virus in humans may take many forms. Such infections are often asymptomatic, but in those who become ill, the disease can range from an uncomplicated fever of unknown origin to a fulminating hemorrhagic disease. Following an incubation period of 7–14 days, onset is typically insidious. As the disease progresses, patients usually develop pharyngitis, which is often purulent and accompanied by headache, fever, myalgia, back or abdominal pain, vomiting, and diarrhea. Despite severe prostration most patients recover spontaneously. Some patients, however, deteriorate rapidly, developing facial and neck edema, respiratory distress, oliguria or anuria, and finally hypovolemic shock that responds poorly to fluid replacement (MCCORMICK et al. 1987a).

Complications commonly involve the CNS and are thought to result mainly from cerebral edema, but possibly confounded by viral encephalitis or intracranial hemorrhage. Acute VIIIth nerve deafness, pleural and pericardial effusions, and clinical signs of myocarditis may also occur. Contrary to impressions from anecdotal descriptions, overt bleeding is uncommon, but when it does occur it may manifest as spontaneous gum bleeding, hematemesis, melena, epistaxis, hematuria, or multiple bleeding sites. Pregnant women with Lassa fever usually abort and may have profuse vaginal bleeding (PRICE et al., submitted). Recovery

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is usually complete, even though convalescence may be prolonged, and wasting and asthenia severe; rarely uveitis and orchitis occur. However, some patients have suffered persistent deafness.

A detailed, prospective case control study in which 31 clinical variables were analyzed in 441 hospitalized patients has been conducted in Sierra Leone. The best combination of sensitivity, specificity, and predictive value for the clinical diagnosis of Lassa fever was seen in the presence of any two of three variables: pharyngitis, purulent pharyngitis, or proteinuria (McCormick et al. 1987a).

2 Pathology of Lassa Fever

The pathology of Lassa fever is remarkably subtle as based on necropsy studies, and the cause of shock or death is not obvious (McCormick et al. 1987b). The most consistent finding in the fatal disease of humans and experimentally infected primates is hepatocellular necrosis. However, this is almost invariably accompanied by evidence of hepatocyte regeneration, and the extent of hepatic damage is insufficient to implicate hepatic failure as the cause of death. Furthermore, there is no correlation between the degree of morphologic liver pathology and biochemical measures of hepatocellular damage or virus titer in the blood or liver. There is no lymphocytic infiltration of necrotic tissue, and although the damage observed is thought to result directly from viral injury (a large quantity of Lassa antigen may in fact be present in the liver), few whole virions are seen using electron microscopy.

3 Lassa Infection in Animals

The natural host of Lassa fever is the African multimammate rat, *Mastomys natalensis* (Monath et al. 1974). Infection of *Mastomys* neonates does not cause disease or pathologic lesions despite abundant virus in blood, lymph nodes, liver, spleen, lung, brain, urine, and throat secretions (Walker et al. 1975). The virus persists in these animals throughout life, presumably having induced host immunotolerance, although there is some evidence that antibodies are produced and that there may be limited virus clearance. There is profuse shedding of virus in urine throughout the life span of the host in the same manner as the closely related lymphocytic choriomeningitis virus in mice.

Experimental Lassa virus infection in adult monkeys and guinea pigs follows an entirely different course more closely resembling that in humans. There is an acute generalized disease, often fulminating and frequently fatal. In guinea pigs the mortality rate is high with marked respiratory symptoms and pathologic evidence of myocarditis, pulmonary edema, and hepatocellular damage (Walker et al. 1975; Callis et al. 1982; Jahrling et al. 1982). Survivors apparently harbor no virus, but develop high antibody titers. In monkeys the infection closely resembles the severest form of Lassa fever in humans and is almost

uniformly fatal. Monkeys have, therefore, been used successfully as an experimental model for studies of the pathophysiology, immunology, and treatment of Lassa fever (CALLIS et al. 1982).

4 Virology and Laboratory Diagnosis

In infected humans and primates, Lassa virus can be recovered from most tissues and body fluids (JOHNSON et al. 1987). Fatality rates in humans are related to virus titer, and for those in whom this exceeds $10^{3.6}$ tissue culture infection dose $TCID_{50}$/ml in serum, the mortality rate is 76%. Titers in tissues may exceed those in the blood, particularly in liver, spleen, lung, kidney, and adrenals. The virus is undoubtedly able to penetrate the CNS since it has been isolated from the cerebrospinal fluid (CSF) of a few patients and from the CSF and brains of monkeys, although titers are generally lower than in blood. Virus can also be recovered from throat swabs, but recovery rates are low and not related to viremia, so this technique has proved unreliable for diagnosis. Viruria is variable, and only detectable in 3% of patients during the acute illness. However, persistent viremia and persistent, though intermittent, viruria have been reported in a very small number of patients. Neither the mechanism nor the timing of virus clearance has been adequately studied in enough humans to establish whether virus persistence is a significant feature of this infection. Techniques to detect the antigen in serum are being developed and, though not yet sufficiently reliable for field evaluation, are likely to become available in the future. Immunofluorescent antibody (IFA) staining with monoclonal antibodies of liver impression smears from human postmortem biopsy material or from livers of trapped rodents seems to be reliable and specific, and is under field evaluation. At present, diagnosis of infection in humans rests on detection of antibodies in serum, or virus isolation.

5 Immune Response

Fatal disease in humans with Lassa fever is related to virus titer but appears not to be related to the timing or level of the antibody response (JOHNSON et al. 1987). Brisk production of Lassa-specific primary IgG antibodies is invariable, and is detectable by immunofluorescent antibodies (IFA) in 53% of patients on admission to hospital. IgM antibodies are present in half of all patients during the first 6 days of illness. Peak IgG titers of 1024 or greater using IFA are reached within 2–3 days of the beginning of IgG production. Despite this antibody response, viremia is unaffected, and virus cocirculates with high titers of antibody, sometimes for 2–3 weeks. Circulating immune complexes have not been detected, and it seems unlikely that they play a role in the pathology of the acute infection. In human sera, neutralizing antibodies cannot be demonstrated at any time in the illness. In monkey sera, however, a neutralization

index can be demonstrated with constant serum (1:10) varying virus dilution format. An index of 2.0 or greater in immune serum administered to monkeys has been correlated with protection against challenge with Lassa virus (JAHRLING and PETERS 1984).

There are no studies evaluating the role of cell-mediated immunity (CMI) in recovery from Lassa infections, virus elimination, or protection against reinfection. Indeed, it seems that reinfections do occur, although it is likely from observations in the field that they are clinically silent (MCCORMICK et al. 1987b). Limited studies in monkeys have shown lymphocyte responses to nonspecific mitogens, suggesting suppression of cell-associated immunity during the acute illness, with transient early lymphopenia primarily due to a decrease in T-suppressor subsets (FISHER-HOCH et al. 1987). Additionally, sharply rising neutrophil counts, preceding the onset of severe illness and shock, have been observed in primates and patients. These cells may be involved in the shock associated with Lassa fever. Circulating neutrophil counts as high as 30000 are recorded with a marked left shift (FISHER-HOCH et al. 1985).

It is reasonable to suggest that recovery of the T-cell populations and subsequent development of a specific cytotoxic T-cell response are eventually responsible for elimination of the virus and for protection against disease during reinfection. There is at present only indirect evidence for this hypothesis.

6 Pathophysiology

Recently, studies of the pathophysiology of Lassa infection in primates and in patients have shown that neither hepatic dysfunction nor disseminated intravascular coagulation is a major component. There is a marked discrepancy in aspartate and alanine transaminase levels, with ratios as high as 11:1, a phenomenon that has been noted in another viral hemorrhagic fever, Marburg disease (FISHER-HOCH et al. 1987). It seems likely that the source of aspartate transaminase is not entirely hepatic. Furthermore, blood glucose levels remain within normal limits, and prothrombin times are rarely abnormal even in the terminal stages (FISHER-HOCH et al. 1985). It is reasonable to conclude that death from Lassa fever is not primarily associated with fulminating hepatitis. Similarly, there is little disturbance in coagulation tests, with prothrombin times only marginally prolonged, and undetectable or low levels of fibrinogen degradation products. Platelet and fibrinogen turnover studies in infected primates show no increase in consumption compared with normal controls. These studies exclude disseminated intravascular coagulation as in important component of the pathology of Lassa fever. If it occurs, it is as a complicating, terminal event. On the other hand, studies of the dynamics of platelet and endothelial cell function in primates and in patients infected with Lassa virus show that both cell systems are functionally impaired despite adequate numbers of circulating platelets and histologically normal endothelium. In primate studies, platelet counts did not fall below normal limits (200000×10^9/liter), whereas in vitro aggregation was abnormal by day 6 postinoculation, and absent by day 13.

In a surviving animal, platelet aggregation rapidly became normal. Similarly, endothelial biopsy specimens taken from moribund primates showed failure of prostacyclin (PGI_2) production, concurrent with the platelet failure. Both of these functions recovered rapidly in a surviving animal.

There has been much recent research on the role of PGI_2 and thromboxane (TXA_2) in shock (LEFER 1985). PGI_2 is the major product of arachidonic acid metabolism in the endothelial cell (VANE 1985). When released locally into the circulation, it causes vasodilation and inhibits platelet adhesion and aggregation by binding with specific surface-platelet membrane receptors and increasing intracellular cAMP concentrations. Conversely, arachidonic acid metabolism in the activated platelet leads to the formation of TXA_2, a vasoconstrictor and a strong inducer of platelet activation and aggregation. These metabolites are highly active but have extremely short half-lives, and are believed to play central roles in maintaining the local homeostasis of the microcirculation.

The disturbance of this homeostatic mechanism in animals has now been studied in patients with Lassa fever (FISHER-HOCH et al., in preparation). The findings suggest a reversible, biochemical injury to platelets and endothelial cells, rather than destruction due to lytic replication by the virus. Failure of the microcirculation, particularly of endothelium, leads to loss of fluids, electrolytes, protein, and other molecules from the intravascular compartment. The hemorrhagic tendency must also be caused by platelets and endothelial cell damage. In most cases adequate reserves in the coagulation cascade system prevent gross hemorrhage. This generalized microcirculation damage may possibly be mediated by effector substances released by neutrophils and macrophages, such as vasoactive amines, complement components, superoxides, or free radicals, all of which can damage membranes and lead to functional disturbances.

7 Treatment

A major trial in Sierra Leone has shown that ribavirin is effective treatment for Lassa fever (MCCORMICK et al. 1986a). Ribavirin is a guanosine analogue with ribose and triazole moieties and acts, at least in part, by competitive inhibition primarily of the guanylation step of the 5′ capping of viral messenger RNA (GOSWAMI et al. 1976; SMITH 1984). However, its antiviral effect is unique in specificity in that any change in its basic structure, such as substitution of any sugar on the triazole carboxamide other than ribose, abolishes its antiviral activity. Similarly, variation in the heterocyclic portion of the molecule also abolishes antiviral properties, despite a wide array of candidates. Nevertheless, some derivatives, such as ribavirin triacetate and some triazole derivatives, do have antiviral action, and may have other advantages such as lipid solubility and, thus, ability to cross the blood-brain barrier (SMITH 1984; HUGGINS et al. 1984).

Ribavirin is metabolized through phosphatization and has a long half-life in humans, especially in red blood cells, probably owing to weak phosphatase activity in them. It may also be sequestered in tissues in the phosphorylated

Table 1. Mortality of Lassa fever patients treated within 6 days of onset of disease

	Admission viremia $\geq 10^{3.6}$ $TCID_{50}$/ml	Admission aspartate transaminase (AST) ≥ 150 IU/liter
No therapy	15/20[a] (75%)	11/18 (61%)
Oral ribavirin	1/5 (20%)	1/5 (20%)
IV ribavirin	1/11 (9%)	1/20 (5%)
Plasma	5/9 (56%)	6/16 (38%)

[a] Number of deaths/total number of patients

Table 2. Mortality of Lassa fever patients treated 7 or more days after onset of illness

	Admission viremia $\geq 10^{3.6}$ $TCID_{50}$/ml	Admission AST ≥ 150 IU/liter
No therapy	21/27[a] (78%)	22/44 (52%)
Oral ribavirin	2/5 (40%)	1/9 (11%)
IV ribavirin	9/19 (47%)	11/43 (26%)
Plasma	7/12 (58%)	8/12 (66%)

[a] Number of deaths/total number of patients

form, particularly in tissues that are also deficient in active phosphatases. Toxicity, manifest mainly as a minor drop in hemoglobin during therapy, is rapidly reversible on stopping the drug, and has not proved to be a problem in patient trials (SHULMAN 1985).

Early studies showed that efficacy of ribavirin against Lassa virus in vitro and in primates (JAHRLING et al. 1984). A patient study has now documented that ribavirin can prevent death in patients with Lassa fever (MCCORMICK et al. 1986a). In this study patients were selected for treatment on the basis of outcome predictors: admission levels of serum AST and viremia. In untreated patients an AST of ≥ 150 IU/liter on admission was associated with an overall case fatality rate of 55%, and viremia of $\geq 10^{3.6}$ $TCID_{50}$/ml on admission with a case fatality rate of 76% (Tables 1, 2). For practical purposes, an AST of ≥ 150 IU/liter on admission was used for selecting patients for treatment, since this can be tested in the field on the day of admission.

Table 1 illustrates the improvement in survival of patients treated with ribavirin within 6 days of onset of disease, compared with untreated patients and patients treated with Lassa convalescent-phase plasma. The greatest improvement was observed in patients with viremias of more than $10^{3.6}$ $TCID_{50}$/ml treated with intravenous ribavirin ($P=0.006$). Table 2 shows similar data for patients in whom treatment was not initiated until 7 or more days after onset. Here improvement in mortality rates was not so dramatic, but the improvement in survival for patients admitted with high viremias and treated with intravenous

ribavirin was still highly significant ($P=0.035$). Although numbers were small, improvement in survival of patients treated with oral ribavirin was also statistically significant ($P=0.008$).

Intravenous ribavirin was administered as a 2-g loading dose, followed by 1 g per day in divided doses, every 8 h for 4 days, and reduced to 0.5 g per day for an additional 6 days. Oral ribavirin was administered in a 2-g loading dose followed by 1 g per day in divided doses, every 8 h for 10 days. Patients receiving immune plasma received 1 unit of Lassa-convalescent plasma with an IFA titer ≥ 128, approximately 4 ml per kg body weight.

Viremias in patients treated with oral ribavirin or with plasma did not differ significantly from those in untreated patients, irrespective of whether they lived or died. Intravenous ribavirin, on the other hand, resulted in marked declines in viremia levels compared with those in untreated patients, regardless of whether they were treated within 6 days of onset or later.

Variable results have been obtained in attempts to treat Lassa fever with plasma from patients convalescent from laboratory-confirmed Lassa fever. Immune-plasma therapy has been successful in treating a related hemorrhagic fever, Argentine hemorrhagic fever (MAIZTEGUI et al. 1979), and results have been encouraging in plasma-treated primates experimentally infected with Lassa virus. The geographic origin of the infecting virus and the infusion of the plasma early in disease both appear to be important (JAHRLING and PETERS 1984); animal studies have shown that the immune plasma must have a high neutralization index. Selection and screening of suitable plasma, and storage and inactivation procedures also present problems. In prospective patient trials in Sierra Leone, convalescent-phase plasma did not improve the outcome in patients with Lassa fever (MCCORMICK et al. 1986a). It seems that the failure of antibody to clear viremia in humans and, indeed, the possibility that it might exacerbate the viral infection, make this therapeutic approach of unproven benefit at present. Concentrated, inactivated human serum with a high neutralization index may be found effective and could be useful in late disease, although such a product would be expensive, and stringent safety procedures would be essential.

In vivo, prostacyclin and its related compounds have been shown to inhibit the activation of platelets and increase microcirculatory blood flow, hence maintaining tissue oxygenation independent of blood flow (LEFER 1985). It can also inhibit neutrophil activation and protect tissues from oxidation damage. A study is now under way to analyze the therapeutic use of prostacyclin in patients with late, severe Lassa fever in whom the prognosis remains poor despite ribavirin therapy.

8 Prevention

A recently completed study has shown that simple barrier nursing techniques utilizing reusable gowns, gloves, and masks are sufficient to prevent nosocomial transmission of Lassa virus to hospital staff (HELMICK et al. 1986). Such evidence suggests that aerosols play little or no role in nosocomial spread. Rather, trans-

mission probably occurs as a result of subcutaneous inoculation, and contamination of cuts, abrasions, or mucosal surfaces. Infections in hospital staff invariably result from failure to observe the basic rules of good barrier nursing, especially use of gloves and disinfectants. Furthermore, most nosocomial infections have been from patients in whom Lassa fever was not suspected and no precautions taken. If an accident occurs involving known subcutaneous or mucosal contamination, postexposure prophylaxis with oral ribavirin should be offered (500 mg three times daily for 7 days).

9 Vaccines

In view of the potential importance of CMI in protection, killed vaccines may not be successful. There are apparently nonpathogenic, Lassa-related viruses that might be considered as candidates for a live vaccine, but it seems improbable that their lack of pathogenicity could be sufficiently guaranteed at the present time to contemplate their use in humans. Lassa virus has a capacity for latency, perhaps even in the human host, with antibody coexisting with virus in the serum. By far the best candidate vaccine is a genetically engineered vaccinia virus expressing viral glycoproteins (AUPERIN et al. 1986). These very recent developments make it possible to envisage human trials of such vaccines in the forseeable future.

10 Conclusion

We now have effective antiviral therapy for Lassa fever with ribavirin. The original frightening accounts of a fulminating, fatal disease must now be relegated to history. Although severe, life-threatening disease occurs, it must be seen against the background of numerous mild and silent infections. Furthermore, awareness of the needs of patients, the now well-defined hazards to staff, and the availability of postexposure prophylaxis should allow active modern management of patients wherever facilities allow. This in itself will reduce the mortality rate even further.

Despite these major advances, there are important areas of future study. The frequency of CNS involvement is not well defined in Lassa fever. The fact that ribavirin may not cross the blood-brain barrier at therapeutic levels means that CNS infection may respond poorly to treatment. A lipid-soluble drug might be of great benefit. We also need more information on minimum dose regimes for effective therapy, and on identification of patients in whom oral therapy will be adequate for control of the disease process. Lassa fever provides an opportunity to study prostaglandin metabolism in severe infection with shock, and a model in which to apply new therapeutic approaches.

Advances in the engineering of vaccines may soon give us candidates for animal protection studies and field trials, and knowledge of the nature and significance of the CMI response in protection will allow us to evaluate their potential. Before long, Lassa fever may not only be treatable, but preventable.

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