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CYTOTOXIC LYMPHOCYTES IN VIRAL HEPATITIS

A Thesis Presented

By

Kim W. McIntyre

Submitted to the Faculty of the
University of Massachusetts Medical School in partial
fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY IN MEDICAL SCIENCES

April 1987

IMMUNOLOGY

CYTOTOXIC LYMPHOCYTES IN VIRAL HEPATITIS

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ABSTRACT

Cytotoxic Lymphocytes in Viral Hepatitis

(April 1987)

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Directed by: Raymond M. Welsh

The immunological mechanisms involved in virus-induced hepatitis were examined by measuring the cytotoxic capabilities and the morphological and antigenic phenotypes of leukocytes isolated from the livers of virus-infected mice. Large granular lymphocytes (LGL) of both natural killer (NK) cell and cytotoxic T lymphocyte (CTL) phenotypes accumulated in livers of mice infected with lymphocytic choriomeningitis virus (LCMV) of either the nonhepatotropic Armstrong strain (LCMV-ARM) or the hepatotropic WE strain (LCMV-WE). NK cell activity and LGL number increased 3- to 4-fold between days 1 and 5 postinfection (p.i.). These LGL were characterized as NK cells on the basis of cell surface antigens, kinetics of appearance, target cell range, and morphology. By day 7 p.i., virus-specific, H-2-restricted, Thy-1⁺, Lyt-2⁺ CTL activity was present in the liver, and its appearance correlated with a second wave of LGL accumulation. Total CTL activity, leukocyte numbers, and CTL/LGL numbers were

at least 5-fold higher in the livers of LCMV-WE-infected mice than in the livers of LCMV-ARM-infected mice. Mice infected with the cytopathic viruses, mouse hepatitis virus and murine cytomegalovirus, experienced greater increases in NK/LGL by day 3 p.i. than did mice either infected with LCMV or injected with poly I:C. The early and late accumulations of LGL in the virus-infected liver were associated with the appearance of two waves of LGL with blast cell morphology expressing the phenotypes of NK cells and CTL, respectively. Thus, the organ-associated accumulation, blastogenesis, and in situ proliferation of cytotoxic LGL provide a means for the localization and site-specific augmentation of a host's cell-mediated antiviral defenses.

The mechanism of inhibition of virus synthesis in vivo by immune splenocytes containing virus-specific CTL was examined in mice dually infected with two different viruses and then adoptively immunized with spleen cells immune to one of the two viruses. Only the titer of the virus to which the splenocytes were immune was reduced in titer, and no nonspecific antiviral effect was seen on the titer of the 'bystander' heterologous virus. These data are consistent with an in vivo mechanism of CTL-mediated antiviral resistance involving direct cytotoxicity rather than release and dissemination of antigen-nonspecific antiviral factors, such as interferon, following recognition of appropriate viral antigen.

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ABBREVIATIONS

ADCC	antibody-dependent cell-mediated cytotoxicity
asialo GM1	ganglio-tetraosylceramide
C	complement
CTL	cytotoxic T lymphocyte
CVF	cobra venom factor
CY	cyclophosphamide
E:T	effector to target
FACS	fluorescence-activated cell sorter
FCS	fetal calf serum
GPC	guinea pig C
HAV	hepatitis A virus
HBSS	Hank's balanced salt solution
HBV	hepatitis B virus
HEPES	N'-2-hydroxyethylpiperazine-N'-ethanesulfonic acid
i.c.	intracerebral(ly)
IFN	interferon(s)
IL-2	interleukin 2
INT	iodophenyl nitrophenyl phenyltetrazolium chloride
i.p.	intraperitoneal(ly)
i.v.	intravenous(ly)
LCMV	lymphocytic choriomeningitis virus
LCMV-ARM	Armstrong strain of LCMV
LCMV-WE	WE strain of LCMV

MCMV	murine cytomegalovirus
MEM	minimal essential medium
MHC	major histocompatibility complex
MHV	mouse hepatitis virus
MIBT	N-methyl-isatin beta-thiosemicarbazone
NANB	non-A, non-B
NK	natural killer
PBL	peripheral blood lymphocyte
PBS	phosphate buffered saline
poly I:C	poly-inosinic:poly-cytidylic acid
PV	Pichinde virus
RbC	rabbit C
T	thymus-derived

CHAPTER I
INTRODUCTION

Cytotoxic lymphocytes are intimately involved in the regulation and eradication of viral infections in mammalian hosts. The particular cytotoxic effector cells responsible for these roles and the extent of their influence may vary among different infections. The reasons for this variation may include such factors as viral cytopathogenicity, route of entry of the virus, tissue tropism(s) of the virus, previous exposure to the infecting agent, as well as the immunological competence, genetic background, and age of the host.

This dissertation examines the involvement of two types of cytotoxic lymphocyte, the natural killer (NK) cell and the cytotoxic T lymphocyte (CTL), in viral infections which cause hepatic disease. The mechanism whereby virus-immune CTL mediate antiviral protection in vivo is probed. The types of cytotoxic effector cells induced to accumulate in the liver following infection with a variety agents of viral hepatitis are characterized phenotypically, functionally, and morphologically. These new data, when correlated with current knowledge of the relative importance of particular cytotoxic lymphocytes in certain viral infections, broaden understanding of the mechanisms of antiviral resistance and immunopathology in viral disease.

A. Cytotoxic T lymphocytes (CTL)

1. The early evidence. Experimental evidence for a direct role of the immune system in providing protection from viral infection came from early studies in mice infected with lymphocytic choriomeningitis virus (LCMV), a natural pathogen of mice (Traub, 1936). The relatively non-specific immunosuppressive effects of x-irradiation or of cytoreductive drugs prevented lethal meningitis in adult mice following intracerebral (i.c.) inoculation of virus (Hotchin and Weigand, 1961; Hannover Larsen, 1969; Gilden et al., 1972a). Similar results were achieved following neonatal thymectomy (Rowe et al., 1963) or in vivo administration of anti-thymocyte serum (Hirsch et al., 1967), implying involvement of a thymus-derived (T) cell. While mice treated in this fashion often did not die during subsequent viral challenge, they generally failed to clear the virus and became chronically, or persistently, infected. It was thus apparent that the virus itself was not directly responsible for death of normal mice, but rather a thymus-dependent component (T lymphocyte?) of the host was responsible for the marked accumulations of inflammatory cells in the central nervous system and the ensuing destruction of neural tissue (Hannover Larsen, 1969; Rowe et al., 1963; Hirsch et al., 1967).

These methods of immunosuppression did not successfully protect mice from all viral infections. Infections caused by vesicular stomatitis virus (Rowe et al., 1963), ectromelia virus

(Blanden, 1970), murine cytomegalovirus (Brody and Craighead, 1974), and vaccinia virus (Hirsch et al., 1968; Worthington, 1973) were decidedly worsened by immunosuppressive treatment, and mortality was usually increased. Thus, in certain instances the immune system seemed to be the cause of disease, while in others it was the cure. Realistic hypotheses explaining this paradox were formulated when it was found that virus-immune leukocytes could preferentially interact with virus-infected target cells in vitro leading to the destruction of the target cells or to inhibition of virus spread (Speel et al., 1968; Lundstedt, 1969; Oldstone et al., 1969; Oldstone and Dixon, 1970; Ennis, 1973; Zinkernagel and Althage, 1977). This effect was variably attributable either to release of cytotoxic factor(s) into the culture supernatant (Speel et al., 1968; Oldstone and Dixon, 1970) or to direct cell-to-cell contact (Oldstone and Dixon, 1970; Ennis, 1973). The development of a quantitative in vitro assay (Brunner et al., 1968) for the activity of killer lymphocytes simplified measurements of this phenomenon. From these findings it was surmised that immune lymphocytes might reduce viral titers in vivo by killing virus-infected cells before progeny viruses were produced. Alternatively, if many cells in a vital organ (such as the brain) were infected, their immune cell-mediated destruction could produce severe pathology or death.

2. H-2 restriction. Demonstrations of cytotoxic T lymphocytes

generated during the course of viral infection were soon to follow for a variety of viruses including ectromelia virus (Gardner et al., 1974a; Gardner et al., 1974b), LCMV (Marker and Volkert, 1973), and Sindbis virus (McFarland, 1974). In 1974, a major advance in immunology was made by the demonstration that LCMV-specific CTL-mediated lysis of LCMV-infected target cells was restricted by antigens of the major histocompatibility complex (MHC), H-2, of the mouse (Zinkernagel and Doherty, 1974). That is, the killer cell had to be generated in a mouse bearing H-2 antigens common with those expressed on the target cell for lysis to occur. The genes coding for these antigens were mapped to the K and D regions (class I genes) of the H-2 complex (Blanden et al., 1975; Zinkernagel and Doherty, 1975). Expression of the class I gene proteins on the virus-infected target cell surface was requisite for a lytic interaction (Zinkernagel and Oldstone, 1976). Increased levels of class I antigen expression, induced by exposure of virus-infected targets to interferon (IFN), enhanced target cell sensitivity to virus-specific CTL-mediated lysis (Bukowski et al., 1985a). This requirement for H-2-restriction was extended to vaccinia virus (Koszinowski and Ertl, 1975), influenza virus (Ennis et al., 1977), Bebaru virus (Mullbacher and Blanden, 1978), and murine cytomegalovirus (Quinnan et al., 1978). Curiously, some strains of mice failed to generate detectable virus-specific CTL activity when infected with certain viruses, and this "defect" in immune

responsiveness often mapped to the class I regions of H-2 (Mullbacher and Blanden, 1978; Rosenthal and Zinkernagel, 1981).

3. Antiviral activity and immunopathology. Volkert (1962) provided some of the earliest evidence that transfer of lymphocytes (adoptive immunization) from virus-immune mice could reduce viral titers in infected recipient mice. Later studies showed that immune T cells were necessary for this antiviral effect (Blanden, 1971) and that T lymphocytes were also important mediators of immunopathology (Cole et al., 1972). By adoptive immunization, in vivo antiviral effects of immune T cells were shown for diseases as diverse as viral meningitis (Gilden et al., 1972b; Hoffsten et al., 1977) and viral pneumonia (Yap and Ada, 1978a; Yap et al., 1979). The in vivo antiviral (Zinkernagel and Welsh, 1976; Yap et al., 1978) and immunopathological (Doherty et al., 1976) activities of transferred immune T cells were shown to be class I antigen-restricted, analogous to the MHC-restriction seen in vitro. Furthermore, a virus-specific, H-2-restricted CTL clone adoptively immunized virus-infected mice (Byrne and Oldstone, 1984). Mutant nude mice (Flanagan, 1966), which lack a thymus (Pantelouris, 1968) and, consequently, thymus-dependent T lymphocytes, were unable to mount a CTL response and were very sensitive to murine cytomegalovirus (MCMV) infection (Starr and Allison, 1977). Most investigators concluded that antiviral protection and/or immunopathology were likely to be mediated predominantly by immune T cells of the CTL subset, although

discrepancies have been noted (Varho et al., 1981; Thomsen et al., 1983; Thomsen and Volkert, 1983; Marker et al., 1985; Pfau et al., 1985).

Virus-specific CTL also accumulated at sites of viral replication in the host during infection. CTL could be found in cerebrospinal fluid during poxvirus (Hapel and Gardner, 1974; Morishima and Hayashi, 1978) and LCMV (Zinkernagel and Doherty, 1973) meningitis, in the peritoneal cavity during viral peritonitis (Pang et al., 1976), and in the lungs during MCMV (Reddehase et al., 1985), influenza virus (Yap and Ada, 1978b; Ennis et al., 1978), and respiratory syncytial virus (Taylor et al., 1985) pneumonia. That virus-specific CTL displayed the same MHC class I-restricted patterns of activity in vitro and in vivo, and that they accumulated at sites of viral replication, further strengthened the argument that it was in fact the CTL that were responsible for mediating antiviral effects in vivo.

B. Natural killer (NK) cells

1. Discovery. At about the time Zinkernagel and Doherty (1974) were unraveling the importance of H-2-restriction in CTL-target cell interactions, other investigators were witnessing a phenomenon of H-2-unrestricted in vitro cytolysis. This killing was reportedly mediated by a Thy-negative (non-T) cell which was present in normal mice (Lamon et al., 1973) and in tumor-inoculated mice (Herberman et al., 1973). These effector cells

were called "natural" killer (NK) cells by Kiessling et al (1975a), since they showed spontaneous cytotoxicity when obtained from unimmunized, naive mice. Because they showed a tendency to preferentially lyse certain virus-induced tumor cells (Lamon et al., 1973; Herberman et al., 1973; Kiessling et al., 1975b), it was postulated that they might provide a first line of defense against tumors arising in vivo. High levels of NK cell activity were found in the spleens of mice, but the activity was low or absent in lymph nodes, bone marrow and thymus (Kiessling et al., 1975a). NK cell activity showed age-dependent variations, being low in neonatal mice, increasing to peak levels between 3-7 weeks of age, and gradually declining in later months (Kiessling et al., 1975a). An intact and functional bone marrow microenvironment was necessary for the development and maintenance of NK cell activity (Haller and Wigzell, 1977; Haller et al., 1977; Kumar et al., 1979). The presence of NK cells in the spleens of nude mice, and the absence (Kiessling et al., 1975a) or low levels (Herberman et al., 1978) of Thy antigen on NK cells from normal mice, suggested that this effector population was truly different from the classical CTL.

2. The NK cell-virus connection. Evidence of non-specific cytotoxic activity, often seen during the early course of viral infections, was reported by other investigators (Blanden and Gardner, 1976; Rodda and White, 1976; Wong et al., 1977; MacFarlan et al., 1977; Herberman et al., 1977). Because the in

vitro pattern of lytic activity resembled that reported by Kiessling et al (1975b) and because the activity was augmented early during viral infection, it was proposed that NK cells might provide an immunologically nonspecific first line of defense against viruses (Welsh and Zinkernagel, 1977; Welsh, 1978b; Welsh et al., 1979).

Augmented NK cell activity during viral infection coincided with the production of interferon (IFN) and preceded the peak of virus-specific CTL activity (Welsh and Zinkernagel, 1977; Welsh, 1978a). This augmented, or "activated", NK cell activity was reflected in an increased ability to lyse targets normally resistant to resting "endogenous" NK cells (Welsh and Zinkernagel, 1977; Welsh et al., 1979). Injections of IFN or IFN-inducers resulted in NK cell activation (Welsh, 1978a; Gidlund et al., 1978; Djeu et al., 1979a; Djeu et al., 1979b), and this activation was blocked by anti-IFN antibody administered in vivo (Gidlund et al., 1978). IFN augmented NK cell activity by recruiting non-lytic pre-NK cells and enhancing the kinetics of NK cell lysis (Silva et al., 1980). IFN-inducers or IFN alone also resulted in the blastogenesis and proliferation of spleen NK cells leading to an increase in their number in vivo (Kiessling et al., 1980; Biron and Welsh, 1982; Biron et al., 1983; Biron et al., 1984). Together, the increased NK cell numbers and increased lytic activity on a per cell basis account for the overall NK cell activation witnessed during viral infection or

IFN exposure.

3. NK cell-mediated antiviral effects. With the demonstrations of NK cell activation during viral infection, a search began for an antiviral role for these cytolytic effector cells and a number of correlations between NK cell activity and antiviral resistance were to follow. Mice with genetically high NK cell activity showed increased resistance to herpes simplex virus (HSV; Lopez, 1981) and MCMV (Bancroft et al., 1981) compared to mice with low NK cell activity. Mice homozygous for the beige mutation, which confers low levels of NK cell activity (Roder and Duwe, 1979), were more sensitive than their NK cell-normal heterozygous littermates to MCMV infection (Shellam et al., 1981). Neonatal mice were susceptible to mouse hepatitis virus (MHV) infection, but developed a natural resistance by 3 weeks of age (Tardieu et al., 1980), coinciding with their age-dependent acquisition of NK cell activity (Kiessling et al., 1975b). Treatments known to augment NK cell activity provided enhanced resistance to subsequent MCMV (Ebihara and Minamishima, 1984) and influenza virus challenge (Spencer et al., 1977). These data were highly suggestive of a role for NK cells in mediating antiviral activity. However, the close relationship between IFN-mediated NK cell activation and IFN's potential direct antiviral effects in the early course of viral infections made it difficult to assign a relative importance to each of these purported resistance mechanisms. Furthermore, these

correlations did not hold for all viruses. LCMV (Welsh and Kiessling, 1980; Allan and Doherty, 1986) and Sindbis virus (Hirsch, 1981) infections were not different in mice homozygous or heterozygous for the beige mutation. Thus, NK cell-mediated resistance did not appear to apply to all viral infections.

The most definitive evidence that NK cells directly provide resistance to at least certain viral infections has come from the work of Bukowski et al (1983b; 1984; 1985). With the development of an antibody reagent directed against the neutral glycosphingolipid ganglio-tetraosylceramide (asialo GM1) present in high concentrations on NK cells, it became possible to selectively deplete NK cell activity in vitro (Kasai et al., 1980) and in vivo (Habu et al., 1981). Pretreatment with anti-asialo GM1 rendered mice more susceptible (i.e., higher organ virus titers and virus-induced tissue pathology) to infection with MHV, MCMV, and vaccinia virus, but not to LCMV (Bukowski et al., 1983b; Bukowski et al., 1984). This increased susceptibility was not due to inhibition of the IFN response to infection as anti-asialo GM1-treated mice had higher IFN titers than infected controls (Bukowski et al., 1983b; Bukowski et al., 1984). Adoptive transfer of NK cell-containing normal adult lymphocytes, or of cloned NK cells, into NK cell-deficient neonatal mice protected them against an otherwise lethal challenge of MCMV (Bukowski et al., 1985). Any treatment which depleted NK cell activity from the transferred donor population

abrogated the cells' in vivo anti-MCMV protective effect (Bukowski et al., 1985). These experiments provided the first direct demonstration of the antiviral activity of NK cells in vivo.

4. Mechanism of NK cell-mediated antiviral action. IFN generated in vivo during early viral infection activates NK cells, making them capable of lysing almost any normal, tumor, or virus-infected target cell (Welsh et al., 1979). If one accepts the proposal that NK cells may inhibit early viral replication by lysing virus-infected target cells in vivo (Welsh et al., 1979), how is wide-spread destruction of surrounding uninfected tissue prevented? Several studies have reported that NK cells may preferentially lyse virus-infected cells compared to uninfected cells (Quinnan and Manischewitz, 1979; Welsh and Hallenbeck, 1980; Piontek et al., 1980; Armerding et al., 1981). This phenomenon was usually seen using endogenous control spleen effector cells in long in vitro assays, conditions which may allow for activation of NK cells by IFN-dependent (Trinchieri and Santoli, 1978) or IFN-independent (Lee and Keller, 1982) mechanisms. In short assays with activated NK cells, such preferential lysis of virus-infected target cells was not the rule (Welsh and Hallenbeck, 1980).

In addition to activating NK cells to kill more efficiently, IFN also has the interesting property of protecting target cells from activated NK cell-mediated lysis (Trinchieri and Santoli,

1978, Welsh et al., 1981; Trinchieri et al., 1981). Since IFN protection requires target cell protein and RNA synthesis (Welsh et al., 1981), infection of that cell by a virus which usurps host cell metabolism would render it insensitive to IFN protection. Cytopathic viruses, like MCMV and MHV, tend to shut down host cell metabolism, while relatively non-cytopathic viruses like LCMV do not. Variation in susceptibility to IFN protection of virus-infected cells may be crucial in determining the extent of NK cell-mediated influence in controlling a particular infection. Thus, an in vivo scenario during viral infection may be hypothesized: 1) virus invades the host, begins replication, resulting in local and systemic IFN production; 2) IFN augments NK cell activity through activation and proliferation; 3) IFN protects uninfected cells and cells infected with non-cytopathic viruses from NK cell lysis; 4) IFN fails to protect cells infected with cytopathic viruses from activated NK cell lysis which can then abort viral replication in those cells. This proposal, and convincing data directly supporting it, have been presented (Trinchieri and Santoli, 1978; Bukowski and Welsh, 1985b).

To exert a direct antiviral effect by cytolysis, NK cells must accumulate locally at a site of viral infection. Several studies have shown NK, or NK-like, cells in cerebrospinal fluid during meningitis of various viral etiologies (Doherty and Korngold, 1983; Moench and Griffin, 1984; Allan and Doherty,

1986). Augmented NK cell activity also developed in the lungs during influenza virus pneumonia (Leung and Ada, 1981; Mann et al., 1985) and in the peritoneal cavity following infection with several different viruses by the intraperitoneal (i.p.), but not the intravenous (i.v.), route (Natuk and Welsh, 1987; Natuk et al., 1987). Both NK cells and CTL thus appear to accumulate at sites of virus infection, a finding readily anticipated from the hypothesis that they mediate antiviral activity by direct cytolytic mechanisms.

C. Large granular lymphocytes (LGL)

Timonen et al. (1979), working with human peripheral blood lymphocytes, drew the first correlations between NK cell activity and the presence of cells morphologically described as large granular lymphocytes (LGL). These LGL displayed relatively abundant cytoplasm, prominent azurophilic cytoplasmic granules, and indented reniform nuclei. This relationship between morphology and function was soon extended to murine NK cells (Luini et al., 1981; Kumagai et al., 1982; Itoh et al., 1982). When granules were purified from NK cell LGL tumors of the rat, they were directly lytic for target cells, implying involvement of the granules in the NK cell lytic process (Millard et al., 1984).

Investigations of T lymphocytes from various sources also revealed the presence of granules in the cytoplasm of cells of

this lineage (Schrader et al., 1983; Podack and Konigsberg, 1984). Furthermore, mouse T lymphocytes maintained in culture often showed an NK cell-like pattern of cytotoxicity, and this was reflected by their LGL morphology (Brooks et al., 1982; Shortman et al., 1983). The granules isolated from in vitro maintained CTL lines were able to mediate target cell lysis (Podack and Konigsberg, 1984; Young et al., 1986b). The lysis appeared to be due to the formation of channels in the plasma membrane of target cells by the polymerization of a protein of the granules, forming structures analogous to the pores formed by components of the complement cascade (Henkart et al., 1985; Podack, 1986; Young et al., 1986a). Evidence was presented that both NK cells and CTL utilize similar pathways in their lytic interactions with target cells (Hiserodt, 1985). The question arose, however, as to whether the granules found in LGL of T cell lineage maintained in vitro were a culture-induced artifact, or were they a true representation of the lytic machinery of normal CTL.

This uncertainty was recently resolved when freshly isolated, in vivo-generated, virus-specific CTL were shown to also exhibit the classical LGL form (Biron and Welsh, 1986; Biron et al., 1986). Although these CTL/LGL were morphologically indistinguishable from NK/LGL, the two cell types could be clearly differentiated on the basis of specific cell surface antigen expression and of the kinetics of their appearance

following viral infection (Biron and Welsh, 1986; Biron et al., 1986). Thus, the proposal that the lytic mechanisms of NK cells and CTL are quite similar (Hiserodt, 1985) is supported by the finding of a common morphology (LGL) and a common function (cytolysis) for these two effector lymphocytes.

D. Cell-mediated immunity in viral hepatitis

One of the most clinically significant and extensively studied causes of human viral hepatitis is hepatitis B virus (HBV). HBV is generally considered to be a relatively non-cytopathic virus. Liver pathology in HBV infection appears to be immune system-mediated with cell-mediated immunity proposed to be the major cause of hepatocyte death (Dudley et al., 1972). HBV-infected patients on immunosuppressive chemotherapy show little clinical evidence of hepatitis until the cessation of treatment when an acute bout of hepatitis may ensue (Galbraith et al., 1975). Similarly, renal dialysis patients with immune dysfunction often show a limited and delayed necrosis of hepatocytes (Nordenfelt et al., 1970). Specific antibody does not appear to significantly contribute to the pathology of acute disease since agammaglobulinemic individuals experience classical HBV infections (Good and Page, 1960; Gelfand, 1974). Rather, antibody may block cell-mediated immune responses in vivo resulting in delayed viral clearance and increased likelihood of developing persistent infection (Mondelli and Eddleston, 1984).

A much higher percentage of B lymphocytes in liver biopsy specimens from patients with a chronic persistent hepatitis, as compared to biopsies from patients with acute hepatitis, supports this hypothesis (Husby et al., 1975; Miller et al., 1977).

Peripheral blood lymphocytes (PBL) from HBV-immune patients showed a selective NK cell-like cytotoxicity for HBV-infected hepatoma cells (Chin et al., 1983) and autologous hepatocytes (Eddelston et al., 1982). Virus-specific CTL, demonstrated in PBL from HBV-convalescing patients, showed preferential recognition of HBV core antigen on the surface of infected hepatocytes (Eddelston et al., 1982; Mondelli et al., 1982). However, chronic HBV patients undergoing prolonged IFN therapy often experience an acute exacerbation of hepatitis with increasing serum transaminases while concomitantly losing demonstrable cytotoxic cells from the blood (Dr. A.L.W.F. Eddelston, personal communication). Thus, in studies using PBL, the data give little insight into the in situ relationship between cytotoxic effector cells and virus-infected hepatocytes.

Histological analysis of human liver biopsies has demonstrated the presence of lymphocytes expressing the T8 antigen of cytotoxic/suppressor cells. These T8⁺ lymphocytes predominated (approx. 75%) over other T lymphocyte subsets in areas of hepatocyte necrosis (Montano et al., 1983; Eggink et al., 1984; Pape et al., 1983). The proportion of T8⁺ lymphocytes in PBL of the same patients was considerably lower (30%),

suggesting a selective accumulation of $T8^+$ lymphocytes in the infected liver. This again raises the question of the validity of correlating immunological events in the liver with PBL function and phenotype (Pape et al., 1983).

Lymphocytes expressing NK cell antigens (Leu-7, HNK-1) occasionally comprised up to 25% of lymphocytes associated with areas of hepatic necrosis in acute HBV hepatitis (Eggink et al., 1984) and acute cytomegalovirus hepatitis (Pape et al., 1983). Furthermore, at the ultrastructural level, lymphocytes with reniform nuclei (Kawanishi, 1977) and cytoplasmic granules (Karasawa and Shikata, 1977) were found in intimate cell-surface contact with necrotic hepatocytes, implying a lymphocyte (LGL?)-mediated, contact-dependent cytotoxicity. Unfortunately, these morphological descriptions provide only a static representation of what must be a highly dynamic process, and one can only infer functional activities of the lymphocytes from their immunophenotypes. Recently, cloned lines of interleukin 2 (IL-2)-dependent CTL were derived from liver biopsies of HBV-infected patients (Hoffmann et al., 1986). Whether these in vitro CTL cell lines accurately reflect the in vivo function and specificity of lymphocytes that accumulate in the liver during viral hepatitis remains to be determined.

Numerous other viruses are capable of causing hepatitis in humans. However, the evidence that cellular immunity plays a significant role in mediating antiviral resistance or causing

immunopathology in any of these diseases is less compelling. Elevated cytotoxicity by PBL for autologous hepatocytes was shown in patients with non-A, non-B (NANB) hepatitis (Poralla et al., 1984; Mondelli et al., 1986). Both T cell-enriched and T cell-depleted populations were cytotoxic in those studies, although some degree of antigen- and/or MHC-restricted specificity appeared operative in one case (Mondelli et al., 1986). Unfortunately, the regions of hepatocellular necrosis in the NANB hepatitis liver did not coincide with the areas of inflammatory cell infiltration, leading to the speculation that pathology was not immune cell-mediated but rather due to direct viral cytopathic effects (Dienes et al., 1982).

Hepatitis A virus (HAV)-infected cells were more readily lysed by normal, non-immune PBL than were uninfected cells (Kurane et al., 1986). This 'preferential lysis' appeared to be due mainly to the in vitro activation of NK cells by IFN generated in the culture wells during the relatively long assay period. Whether a similar mechanism occurs in vivo during HAV infection is unknown. A significant level of serum IFN (8 U/ml) was found in only 1 of 12 patients with acute HAV infection and in only 1 of 39 other patients with acute HBV and NANB (Pugliese et al., 1984), suggesting that IFN-activation of NK cells and NK cell-mediated cytotoxicity may not be of clinical significance in these disease processes.

In certain other human hepatitis virus infections, cell-

mediated immunity does not appear to be directly responsible for the induction of liver immunopathology, since little inflammatory cell infiltrate can be seen, even at the height of disease. Rather, viral cytopathic effects seem more damaging to host tissue. Some of the agents which comprise this class include yellow fever (Howard et al., 1984), Ebola (Baskerville et al., 1978), Congo-Crimean hemorrhagic fever (Baskerville et al., 1981), and Lassa fever viruses (Winn and Walker, 1975). These viruses may replicate lytically in lymphoid cells (Wheelock and Edelman, 1969), resulting in direct immunosuppression and lymphoid depletion, thereby crippling the ability of the host to mount a strong cell-mediated response. Aggressive antiviral or immunopotentiating intervention early in infection might grant the host time to generate sufficient cellular responses to combat hepatitis viruses of this type. Studies of animal models infected with attenuated or less virulent strains of some of these viruses may allow an elucidation of the crucial components of the cellular immune system that must be preserved and promoted to improve the prognosis of patients afflicted with these potentially lethal infections.

CHAPTER II

MATERIALS AND METHODS

A. Animals

C57BL/6J (H-2^b) and BALB/cJ (H-2^d) male mice were purchased from Jackson Laboratories, Bar Harbor, ME. C57BL/6J (H-2^b) breeding stock (Jackson Labs) carrying the beige mutation (bg) were bred and maintained in our facilities. C3H/St male mice were purchased from West Seneca Farms, West Seneca, NY. BALB/cAnBOM athymic nude (nu/nu) mice and euthymic nu/+ littermates, derived from breeding stock generously provided by Dr. J. Ware (Duke University, Durham, NC), were bred in our facilities and maintained in sterilized cages with autoclaved food and bedding and acidified water. BALB/cJ mice, congenitally infected with LCMV (Traub, 1936), were bred in our biocontainment facilities for infectious agents. Mice used in experiments were between 6-12 weeks of age unless otherwise specified.

Hartley strain guinea pigs (GP) were obtained from Charles River Laboratories, Wilmington, MA.

B. Cells and Media

L929 cells, a liver cell line from C3H mice (H-2^k), MC57G cells from C57BL/6 mice (H-2^b), and Vero (African green monkey kidney) cells were grown in monolayer culture in Eagle's minimal essential medium (MEM; GIBCO, Grand Island, NY) supplemented with

10% heat-inactivated (56 °C, 30 min) fetal calf serum (FCS; M.A. Bioproducts, Walkersville, MD), 2 mM glutamine, and penicillin/streptomycin (MEM assay medium). YAC-1 cells (Fenyo et al., 1968; Kiessling et al., 1975a), derived from a Moloney leukemia virus-induced lymphoma in A/Sn mice (H-2^a), and K562 cells derived from a patient with chronic myelogenous leukemia (Lozzio and Lozzio, 1975), were maintained in stationary suspension culture in RPMI-1640 medium (GIBCO) with the above supplements (RPMI assay medium).

MEM assay medium and RPMI assay medium from which the FCS had been omitted were termed MEM wash medium and RPMI wash medium, respectively.

C. Viruses

The hepatotropic WE strain (Welsh, 1984b) of LCMV (LCMV-WE), the nonhepatotropic Armstrong strain (Welsh, 1978a) of LCMV (LCMV-ARM), and Pichinde virus (PV), an arenavirus related to LCMV (Buchmeier et al., 1981) were stocks grown in L929 cells (Welsh et al., 1976). Mouse hepatitis virus, strain A59 (MHV; Manaker et al., 1961) was prepared as a 10% homogenate of infected liver tissue in MEM that was clarified by centrifugation (1500 g, 20 min, 4 °C), aliquoted, and stored at -70 °C. Murine cytomegalovirus, Smith strain (MCMV; Smith, 1956), was a salivary gland-grown stock prepared as described for MHV from tissues of BALB/cJ mice at 2-3 weeks p.i. Vaccinia virus, strain WR (Natuk

and Holowczak, 1985), was propagated in Vero cells.

D. Reagents

Poly-inosinic:poly-cytidylic acid (poly I:C), iodophenyl nitrophenyl phenyltetrazolium chloride (INT), and metrizamide were purchased from Sigma Chemical Co., St. Louis, MO. Hank's balanced salt solution without Ca^{++} or Mg^{++} (HBSS) and Gey's solution were obtained from GIBCO. Cobra venom factor (CVF) was purchased from Cordis Laboratories, Miami, FL. N-methyl-isatin beta-thiosemicarbazone (MIBT) was a gift from Dr. C. Pfau, Rensselaer Polytechnic Institute, Troy, NY. Purified mouse IFN-beta was purchased from Lee Biomolecular Laboratories, San Diego, CA.

E. Antisera and Complement

Rabbit antiserum to ganglio-tetraosylceramide (asialo GM1; Wako Chemicals, Dallas, TX) was used to selectively deplete NK cell activity in vivo and in vitro (Habu et al., 1981; Bukowski et al., 1983b). For in vivo use, anti-asialo GM1 serum was diluted 1:10 in RPMI 1640 medium, and 0.2 ml was injected either i.p. or i.v.. Anti-asialo GM1 was used in vitro at a dilution of 1:50. Monoclonal anti-Thy-1.2, clone HO-13-4 (Ledbetter and Herzenberg, 1979) (diluted 1:1500), and J11d antibody (Bruce et al., 1981) (used undiluted) were provided by Dr. R. Woodland (University of Massachusetts Medical Center). Rat monoclonal

antibody to Lyt-2, clone 53-6.7 (Ledbetter and Herzenberg, 1979), was used undiluted. Rabbit serum (RbC), obtained from Pel-Freeze Biologicals, Brown Deer, WI, was used as a source of complement (C). Fresh guinea pig serum (GPC) was prepared from clotted whole blood obtained by cardiac puncture of ether-anesthetized guinea pigs and was used as an alternative source of C. Guinea pig anti-LCMV serum was prepared from virus-immune guinea pigs and was heat-inactivated (56 °C, 30 min) before use. Mouse anti-LCMV serum was obtained from LCMV-immune mice. Guinea pig anti-PV serum was a gift of Dr. M. Buchmeier (Scripps Clinic and Research Foundation, La Jolla, CA).

For in vitro antibody and C depletion, effector cells (see below) were treated with antibody reagent for 30-45 min at 4 °C and then washed. Cells treated with anti-Lyt-2 were further treated (to improve C fixation) with a 1:50 dilution of monoclonal mouse anti-rat immunoglobulin, MAR 18.5 (Lanier et al., 1982), for an additional 30 min at 4 °C and then washed. All cells were resuspended in C for 30 min at 37 °C, washed, resuspended to equal volume, and used in cytotoxicity assays and for the preparation of cytocentrifuge smears for differential leukocyte counts.

F. Injections

Intraperitoneal (i.p.) injections were performed without anesthesia. Mice were injected i.v. by either of two routes:

injections via the lateral tail veins were aided by gently heating the tail with a heat lamp to dilate the vessel; some mice (ether-anesthetized) were injected i.v. via the retro-orbital plexus. Mice injected with infectious agents were housed in the Biocontainment Facility at UMMC. Control, endogenous mice were maintained in conventional housing quarters.

G. Preparation of Effector Cells

1. Spleen leukocytes. Spleen cells were obtained by gently dissociating aseptically removed spleens between two frosted glass microscope slides. Erythrocytes were lysed by treatment with 0.84% ammonium chloride for 5 min. The leukocytes were washed in RPMI wash medium, and the number of viable (0.1% trypan blue-excluding) cells was determined by hemocytometer counts. The cells were resuspended in RPMI assay medium at the desired concentration.

2. Liver leukocytes. Effector cells were prepared from livers following in situ enzyme perfusion (David et al., 1975) with modifications. Mice were anesthetized with sodium pentobarbital (1.0 - 1.5 mg, i.p.), or were decapitated and exsanguinated. The abdominal cavity was opened by a ventral midline incision, and the intestines were lifted to expose the portal vein. A silk suture ligature was loosely placed around the portal vein 0.5 cm from the liver. The portal vein was then catheterized with polyethylene tubing (0.58 mm i.d., 0.965 mm o.d.; Becton

Dickinson, Parsippany, NJ), the ligature was tightened, and the abdominal aorta was transected. Perfusion (2.5 ml/min, 5 min) was begun with heparinized (10 U/ml) Ca^{++} - and Mg^{++} -free HBSS at 37°C. This initial perfusion served to flush peripheral blood from the organ. Only livers that were well-blanching were used for further study. The perfusate was changed to pre-warmed HBSS containing 0.05% (w/v) collagenase (Type 1; Sigma) and 0.05% (w/v) neutral protease (dispase, Grade II; Boehringer Mannheim, Indianapolis, IN), and the perfusion was continued for an additional 10 min. The liver was then excised and teased apart with forceps in HBSS containing deoxyribonuclease (100 U/ml, Type 1; Sigma) on ice for 15 min. The liver cell suspension was passed through nylon mesh to remove clumps and pelleted at 1000 g. The pellet was resuspended in minimal volume (0.5-0.75 ml) and mixed with isotonic 30% metrizamide in Gey's solution at a ratio of 7 parts metrizamide solution to 5 parts liver cell suspension (Wiltrout et al., 1984). This mixture was transferred to a centrifuge tube, overlaid with 2 ml phosphate buffered saline (PBS), and centrifuged for 20 min at 1500 g at 10°C. The non-parenchymal cell layer was harvested from the interface, washed in RPMI wash medium, counted, and resuspended to the desired concentration in RPMI assay medium. This procedure routinely yielded at least $2-5 \times 10^6$ non-parenchymal cells per liver from control mice. In the experiments presented, contaminating hepatocytes accounted for less than 1% of the total

cells recovered.

Plastic non-adherent liver leukocytes were used in some experiments. They were obtained by incubating leukocytes (5 - 7 ml at $2 - 4 \times 10^6$ /ml RPMI assay medium) in 100 mm petri dishes for 1 hr at 37 °C. Nonadherent cells were recovered by aspiration and washing of the plates.

H. Cytotoxicity assay

Target cells (10^6) were labeled with 100 uCi sodium chromate (^{51}Cr ; New England Nuclear, Boston, MA, or Amersham Corp., Arlington Heights, IL) for 1 hr at 37°C, washed, and mixed with effector cells in quadruplicate in round-bottom microtiter wells (Costar, Cambridge, MA) at 10^4 target cells per well in a total volume of 0.2 ml RPMI assay medium supplemented with 10 mM N'-2-hydroxyethylpiperazine-N'-ethanesulfonic acid (HEPES). For spontaneous release determinations medium was added without effector cells. Nonidet P-40 (1%; Sigma) was added at the end of the assay for maximum release determinations. The plates were incubated for 4-16 hr at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. At the end of incubation, plates were centrifuged at 500 g for 5 min, and 0.1 ml of the supernatant was removed and counted in a Beckman gamma counter (Model 5500; Beckman Instruments, Palo Alto, CA). For the data presented, the coefficient of variation among quadruplicate wells was less than 5%.

Data are expressed as % specific ^{51}Cr release (or % specific lysis):

$$\frac{[(\text{cpm experimental} - \text{cpm spontaneous}) / (\text{cpm maximum} - \text{cpm spontaneous})] \times 100}{}$$

In some experiments examining CTL activity, lysis is expressed as: % virus-specific lysis = % specific lysis of virus infected target minus % specific lysis of uninfected target.

To compare the total lytic capabilities of effector populations isolated from different organs, calculations of total lytic units were computed by the formula: total lytic units = (fraction of targets lysed x number of cells per organ) / effector:target ratio. An effector:target (E:T) ratio on the linear portion of the cytotoxicity curve was used for these calculations.

I. Virus plaque assays

Organs were aseptically removed from mice at various times p.i. Spleens were homogenized in 1 ml RPMI assay medium. Livers were weighed and then homogenized in 5 ml RPMI assay medium. Tissue homogenates were clarified by centrifugation at 1500 g at 4 °C for 20 min. The supernatant was collected and titrated for infectious virus immediately or aliquoted and stored at -70 °C until use.

LCMV and PV were titrated by plaque assays on Vero cells. Plastic 60 mm petri dishes (Costar) with confluent cell monolayers were infected with 100 ul of 10-fold serial dilutions

of blood or tissue homogenate in RPMI assay medium. The virus was adsorbed for 1-2 hr at 37 °C in a total volume of 1 ml with occasional agitation of the dishes. The dishes were then overlaid with 4 ml of a 42-45 °C 0.5% agarose solution, prepared as follows: Agarose (type SeaKem HGT, FMC Corp, Rockland, ME) (1%, w/v) was boiled in double-distilled water until dissolved, then cooled to approximately 65 °C. To this was added an equal volume of 2X concentrated MEM with 2X glutamine, 2X antibiotics, 100 U mycostatin (GIBCO)/ml, and 1X FCS. After allowing the agarose to solidify at room temperature, the dishes were incubated at 37 °C in a humidified atmosphere of 5% CO₂ / 95% air for 4-5 days. The plaques were visualized by staining with the addition of 1 ml of 0.5% agarose solution containing 0.01% neutral red dye.

To discriminate between LCMV and PV in tissues of mice dually infected with both viruses, PV was titrated following neutralization of LCMV with GP anti-LCMV serum. This was accomplished by incubating 50 ul of clarified tissue homogenate with 50 ul of a 1:4 dilution of heat-inactivated (56 °C, 30 min) GP anti-LCMV serum for 45 min at 37 °C. Control tubes received 50 ul RPMI assay medium instead of GP anti-LCMV serum. Following incubation, the contents were diluted to 0.5 ml with RPMI assay medium and titrated as above. LCMV was titrated in the presence of PV by staining plaque assay plates with 1 ml of iodophenyl nitrophenyl phenyltetrazolium chloride (INT; 1.5 mg/ml) in 0.9%

NaCl. This dye stains uninfected and PV-infected cells but not LCMV-infected cells (Logan et al., 1975), thus LCMV plaques appear clear on a stained background.

The titers of LCMV and vaccinia virus were discriminated in tissues of dually infected mice by preparing plaque assay plates essentially as described above. One set of plates was stained with neutral red after 2 days incubation for the enumeration of vaccinia virus plaques. N-methyl-isatin beta-thiosemicarbazone (MIBT; 20 ug) was added at days 0 and 2 of incubation to the 4 ml agarose overlay of the second set of plates to inhibit vaccinia virus replication. LCMV plaques were demonstrated by staining the plates after 4 days incubation.

J. Generation of alloreactive CTL

Lymph node cells (5×10^7) from normal BALB/cJ mice were injected i.v. into C3H/St mice irradiated 1 hr earlier with 750 R gamma irradiation. The spleens of recipient C3H/St mice were harvested 5 days later and processed for effector cells as described above.

K. Surgical procedures

Mice anesthetized by injection of sodium pentobarbital (1.0 - 1.5 mg, i.p.), were shaved and then swabbed with ethanol and iodine. A lateral incision was made through the abdominal wall. In beta irradiation experiments (see below), the spleen was

exteriorized on its pedicle, wrapped in sterile gauze moistened with saline, and immobilized. Following irradiation, the spleen was replaced in the abdominal cavity, and the incision was closed with stainless steel surgical staples.

For splenectomy, mice were similarly treated except that following exteriorization of the spleen, a silk suture ligature was tied around the pedicle and the spleen was excised. Sham-operated mice underwent the same surgical treatment but without the application of the ligature or removal of the spleen.

L. Irradiation of mice

Gamma irradiation was administered to mice in a Gammacell 40 (Atomic Energy of Canada Limited, Ottawa, Canada), equipped with twin ^{137}Cs sources, at a dose rate of 125 R/min.

Electron irradiation (20 MeV) was delivered by a Saturne 20 linear accelerator (Thomson-CGR, Columbia, MD) at 300 R/min. Shielding from electron irradiation was provided by inverted 5 mm-thick lead cups. Dosimetry measurements, conducted at the time of irradiation with ionization chambers (PTW Corporation, Freiburg, FRG), indicated that the lead shielding was >95% effective in blocking exposure to electron irradiation.

M. Fluorescence-activated cell sorter (FACS)

Liver leukocytes were stained for FACS analysis and sorting using rat monoclonal antibody 53-6.7 against Lyt 2 followed by

F(ab')₂ mouse anti-rat immunoglobulin conjugated with fluorescein isothiocyanate (JacksonImmunoResearch Labs, Inc., Avondale, PA). Analysis and sorting were performed on a FACS 440 (Becton-Dickinson FACS Systems, Mountain View, CA) at a laser output of 300 mW at 488 nm with green fluorescence collected through a DF 530/30 filter. Cells were separated into fluorescence-negative and fluorescence-positive populations at a flow rate of 2 - 2.5 x 10³ cells/sec.

N. Single cell cytotoxicity assay

To directly visualize cytolysis mediated by dividing cells, a single cell cytotoxicity assay (Grimm and Bonavida, 1979), modified to include autoradiography (Biron and Welsh, 1982), was employed. Liver leukocyte effectors cells were prepared as described above and given to Dr. C.A. Biron and K.F. Pedersen who kindly performed the assay as described (Biron and Welsh, 1982). Six hundred to 700 conjugates that contained a single effector cell and a single target cell were scored per assay. The total percent cytotoxicity is equal to the fraction of single cell conjugates that contained dead target cells X 100. The percent killing mediated by (³H-thymidine-incorporating) blast cells is equal to (the number of single cell conjugates containing dead target cells bound to blast cells / the total number of single cell conjugates containing dead target cells) X 100. Viability of the K562 target cells without added effector cells was greater

than 95%.

O. Centrifugal elutriation

Leukocytes were size-separated using a Beckman JE-6B elutriation centrifuge (Beckman Instruments) essentially as described (Kiessling et al., 1980; Biron and Welsh, 1982). Leukocytes were loaded into the rotor spinning at 3200 rpm. Fractions of increasing size (fractions 1 through 6, respectively) were eluted in HBSS (with Ca^{++} , Mg^{++} and 1.5 % FCS) at flow rates of 15, 22, 28, 32, 38, and 48 ml/min at 4 °C.

P. Leukocyte morphology

One to 3×10^4 leukocytes were centrifuged (650 rpm, 5 min) onto glass microscope slides in a cytocentrifuge (Shandon Southern Instruments, Inc., Sewickey, PA), air-dried, and stained with Wright's and Giemsa stains. Two hundred to 500 leukocytes were counted under 100X oil immersion microscopy for the enumeration of large granular lymphocyte (LGL) percentages. The proportion of LGL exhibiting blast cell morphology (enlarged cell size, basophilic cytoplasm, and/or prominent nucleoli (Hayry and von Willebrand, 1981)) and of mitotic figures with characteristic LGL cytoplasmic granules was determined by counting 100-200 LGL and classifying them into blast or non-blast LGL subsets.

Q. Histological methods

The livers of mice were perfused in situ, using the technique outlined above, with heparinized (10 U/ml) HBSS containing Ca^{++} and Mg^{++} for 5 min at 2.5 ml/min. For paraffin-embedded techniques, the livers were fixed by perfusion for 10 min with 10% neutral buffered formalin. The tissue was processed by conventional techniques, embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E). For immunofluorescence studies, small blocks (approx. 2 x 2 x 5 mm) of spleen tissue were mounted in O.C.T. compound (Miles Scientific, Naperville, IL) and snap-frozen in liquid nitrogen. Four micron serial frozen sections were fixed briefly in acetone then air-dried. They were post-fixed for 10 min in cold acetone and washed in PBS before immunostaining.

R. Statistical analyses

Data are presented as the mean \pm standard deviation, except for data obtained from pooled samples. Statistical significance between groups was determined by Student's t test or the Fisher exact test.

CHAPTER III

ROLE OF NK CELLS IN LCMV INFECTION

A. Acute infection

Considerable evidence has accumulated to date that NK cells play little or no role in mediating antiviral effects during acute LCMV infection (reviewed in Welsh, 1984b). Most of this evidence has been acquired following infection by the Armstrong strain of LCMV (LCMV-ARM). It was of interest to determine whether this apparent lack of NK cell involvement in acute LCMV infection would also apply to infection with another LCMV strain, the hepatotropic WE strain (LCMV-WE). While LCMV-ARM grows only to low titers ($<3 \log_{10}$ PFU/gm) in the liver during acute infection (Welsh, 1984b), LCMV-WE replicates quite well there, yielding titers 100- to 1000-fold higher by day 3 p.i.

Mice were depleted of NK cells by injection of anti-asialo GM1 serum and then infected with LCMV-WE. As shown in Table 1, LCMV-WE grew to comparable titers in the livers and spleens of all mice at day 3 p.i., regardless of their NK cell status. Thus, the replication of LCMV-WE does not appear to be under regulation by NK cells, in agreement with previous work on LCMV-ARM (Bukowski et al., 1983b; Bukowski et al., 1985).

B. Persistent infection

Mice persistently infected with LCMV-ARM have been shown to

Table 1
Effect of Anti-Asialo GM1 Treatment
In Vivo on LCMV-WE Titers During Acute Infection^a

<u>Treatment</u> ^b	% YAC-1 <u>Lysis</u> ^c	<u>Log₁₀ PFU per</u>	
		<u>Spleen</u>	<u>gm Liver</u>
None	21. ± 1.7	6.1 ± 0.1	4.7 ± 0.7
Anti-asialo GM1	-0.2 ± 2.1	6.1 ± 0.1	4.9 ± 0.5

^aNine week old female C57BL/6J mice (n=4 per group) were infected i.p. with 1×10^5 PFU LCMV-WE. Organs were collected at day 3 p.i. and infectious virus titrated.

^bSome mice were treated i.v. 4 hr before infection with 0.2 ml anti-asialo GM1 serum (1:10 dil).

^cYAC-1 assays with spleen effector cells were run for 4 hr at E:T=50:1.

have elevated levels of NK cell activity and elevated levels of circulating IFN (Bukowski et al., 1983a). To determine whether NK cells in persistently infected mice might be exerting an antiviral effect, anti-asialo GM1 serum was administered in vivo to deplete NK cell activity, and the titers of infectious LCMV in various organs were determined 3 days later (Table 2). NK cell activity was significantly reduced ($p < .01$) by antiserum treatment, but the depletion of NK cells had no significant effect on LCMV titers in blood, spleen, or liver (note that LCMV-ARM grows well in liver during persistent infection in contrast to acute infection). Since persistently infected mice make an antiviral antibody response (Oldstone and Dixon, 1967), these data also suggest that NK cells do not regulate, via their Fc receptors, the persistent LCMV-ARM infection by antibody-dependent cell-mediated cytotoxicity (ADCC).

C. Passive immunization of LCMV-infected mice

While NK cells were not apparently directly involved in mediating anti-viral resistance either in acute LCMV-WE infection (Table 1) or in persistent LCMV-ARM infection (Table 2), the possibility existed that an anti-LCMV activity of NK cells, mediated by ADCC, might be demonstrated in acute primary infection. Normal mice were passively immunized with LCMV-immune serum prior to infection. LCMV-immune serum was titrated in vivo for its ability to inhibit subsequent LCMV-ARM infection

Table 2
Effect of Anti-Asialo GM1 Treatment
In Vivo on LCMV Titers in Persistently Infected Mice^a

<u>Treatment</u> ^b	% YAC-1 <u>Lysis</u> ^c	Log ₁₀ PFU per		
		<u>Spleen</u>	<u>gm Liver</u>	<u>gm Brain</u>
None	27. ± 5.0	5.6 ± 0.3	6.2 ± 0.9	4.9 ± 0.5
Anti-asialo GM1	8.1 ± 4.1	5.8 ± 0.3	6.5 ± 0.2	5.0 ± 0.1

^aBALB/cJ mice (n=3 per group) congenitally infected with LCMV, were used at 18 weeks of age.

^bSome mice were injected i.v. with 0.2 ml anti-asialo GM1 serum (1:10 dil) 3 days before organs were harvested and titrated for infectious virus.

^cYAC-1 assays with spleen effector cells were run for 16 hr at E:T=50:1.

(Table 3). A dose of 0.1 ml LCMV-immune mouse serum was shown to significantly ($p < .05$) reduce LCMV spleen titers at day 3 p.i. This dose of antiserum was used in the following experiments.

D. ADCC in acute LCMV infection

As shown in two separate experiments in Table 4, anti-LCMV serum alone significantly reduced day 3 p.i. LCMV spleen titers compared to infected controls ($p < .001$ and $p < .01$, Expt. 1 and 2, respectively). Depletion of NK cells by anti-asialo GM1 serum treatment concomitant with anti-LCMV serum injection yielded day 3 p.i. LCMV spleen titers that were not significantly different than those in mice receiving anti-LCMV serum alone ($p > .10$). Note that infected mice receiving anti-asialo GM1 alone had virus titers that were not significantly different than titers in infected controls. These data suggest that NK cells do not significantly contribute directly, or indirectly via ADCC, to host anti-viral resistance to LCMV.

A very potent, though non-specific, means for suppressing in vivo host immune function is treatment with cyclophosphamide (CY). To examine whether a CY-sensitive component of the host's immune system was interacting with antibody to lower viral titers in this passive immunization protocol, mice were pre-treated with CY then passively immunized and subsequently infected with LCMV. Table 5 shows that while anti-LCMV serum again partially protected mice from LCMV infection, no significant portion of

Table 3
 Titration of Mouse Anti-LCMV
 Serum In Vivo During Acute LCMV Infection^a

Antiserum <u>Injected (ml)</u> ^b	Log ₁₀ PFU <u>per Spleen</u>
None	5.1 ± 0.3
0.10	3.1 ± 1.0
0.05	4.9 ± 0.2
0.025	4.9 ± 0.4

^aEleven week old C3H/St mice (n=3 per group) were infected i.p. with 8×10^4 PFU LCMV-ARM 3 days before organs were harvested for titration.

^bVolume of mouse anti-LCMV serum diluted to 0.2 ml and injected i.v. at the time of infection.

Table 4
 Effect of Anti-Asialo GM1 and Anti-LCMV
 Sera In Vivo on Acute LCMV Infection

<u>Expt</u>	<u>Strain</u> ^a	<u>Anti-LCMV</u> ^b	<u>Anti-Asialo GM1</u> ^c	<u>% YAC-1 Lysis</u> ^d	<u>Log₁₀ PFU per Spleen</u>
1	C3H/St	-	-	52. ± 2.8	5.2 ± 0.3
		+	-	52. ± 5.9	3.4 ± 0.5
		-	+	13. ± 4.9	5.6 ± 0.3
		+	+	26. ± 7.5	4.0 ± 0.8
2	C57BL/6J	-	-	26. ± 5.3	5.3 ± 0.4
		+	-	38. ± 5.7	4.1 ± 0.3
		-	+	0.7 ± 2.2	5.1 ± 0.4
		+	+	2.5 ± 3.0	4.1 ± 0.5

^aC3H/St mice (n=4 per group); C57BL/6J mice (n=4 per group).

^b0.1 ml mouse anti-LCMV serum i.v. at the time of infection.

^c0.2 ml anti-asialo GM1 serum i.v. 4 hr before virus infection.

^dYAC-1 assays with spleen cell effectors were run for 16 hr at E:T=25:1 (Expt. 1) or 4 hr at E:T=50:1 (Expt. 2).

Table 5
 Effect of Cyclophosphamide (CY) and
 Anti-LCMV Serum on Acute LCMV Infection

<u>Expt</u>	<u>Strain</u> ^a	<u>Anti-LCMV</u> ^b	<u>CY</u> ^c	<u>% YAC-1</u> <u>Lysis</u> ^d	<u>Log₁₀ PFU</u> <u>per Spleen</u>
1	C3H/St	-	-	51.	5.5 ± 0.1
		+	-	35.	3.9 ± 0.3
		-	+	7.2	5.0 ± 0.2
		+	+	5.4	4.3 ± 0.2
2	C57BL/6J	-	-	42.	5.4 ± 0.5
		+	-	39.	4.5 ± 0.4
		-	+	15.	5.3 ± 0.3
		+	+	18.	4.6 ± 0.1

^aC3H/St mice (n=3-5 per group) were 10 weeks of age and C57BL/6J mice (n=3-5 per group) were 9 weeks of age.

^bSee "b" Table 4.

^cCY (300 mg/kg, Expt. 1; 200 mg/kg Expt. 2) was injected i.p. 48 hr before infection.

^d16 hr at E:T=25:1 (Expt. 1) or 4 hr at E:T=100:1 (Expt. 2).

this protection was CY-sensitive (compare anti-LCMV vs anti-LCMV + CY). In Expt. 1, mice treated with CY alone showed slight, but statistically significant ($p < .01$), reductions in LCMV titers at day 3 p.i. This effect of CY was not seen in Expt. 2, possibly because a lower dose of CY was employed in Expt. 2. Note that CY-treated mice had considerably reduced NK cell activity, providing further evidence against direct NK cell involvement in LCMV infection.

Welsh et al (1976) showed that the addition of an active source of complement (C) to antibody-coated LCMV virions led to augmented in vitro inactivation of viral infectivity. The in vivo interactions of passively transferred anti-LCMV antibody with host C and host CY-sensitive components were examined by treating mice with various combinations of anti-LCMV serum, CY, and/or cobra venom factor (CVF), which prevents complete activation of the C system by depleting the third component of C (C3) (Muller-Eberhard et al., 1966; Shin et al., 1969). Table 6 shows that pretreatment of LCMV-infected mice with CVF alone (Group D) had no significant effect on LCMV spleen titers at day 3 p.i. compared to infected controls (Group A). However, if passively immunized mice were pretreated with CVF (Group F), some of the protective effect of anti-LCMV serum alone (Group B) was lost ($p < .05$). This suggests that at least a portion of the antiviral effect mediated by immune serum in vivo involved binding and (presumed) activation of C components, by either

Table 6
 Effect of Cyclophosphamide (CY), Cobra Venom
 Factor (CVF) and Anti-LCMV Serum on Acute LCMV Infection^a

Group	Treatment			% YAC-1 Lysis ^e	Log ₁₀ PFU per Spleen
	Anti-LCMV ^b	CY ^c	CVF ^d		
A	-	-	-	18.	5.5 ± 0.3
B	+	-	-	22.	3.5 ± 0.5
C	-	+	-	8.1	4.6 ± 0.3
D	-	-	+	28.	5.6 ± 0.2
E	+	+	-	9.4	3.6 ± 0.5
F	+	-	+	30.	4.4 ± 0.5
G	-	+	+	16.	4.3 ± 0.1
H	+	+	+	10.	3.7 ± 0.7

^aC57BL/6J mice (n=4 per group; except group G, n=3) were 9 weeks old.

^bSee "b" Table 4.

^cCY (300 mg/kg) was injected i.p. 48 hr before infection.

^dCVF (15 U) was injected i.p. 4 hr before infection, and an additional 10 U injected i.p. at day 2 p.i.

^eYAC-1 assays with spleen cell effectors were run for 4 hr at E:T=50:1.

antibody-coated virions or antibody-coated, virus-infected cells.

As seen previously (Table 5, Expt. 1), CY pretreatment alone (Group C) caused a modest, yet significant ($p < .01$), reduction in viral titers compared to controls. The effects of CY and anti-LCMV serum were not additive, however, as viral titers in the presence of anti-LCMV serum without (Group B) or with CY (Group E) were not different.

The antagonistic effect of C depletion on antiserum protection could be overcome by the addition of CY to the treatment regimen (Group H). Under these conditions (C-depletion), the effects of CY and anti-LCMV serum appeared approximately additive (compare Groups C, F, and H). The effect of CVF plus CY (Group G) was not different than CY alone (Group C), suggesting a lack of C involvement in the CY-induced inhibition of LCMV synthesis.

The studies presented in this chapter provide further evidence to substantiate the contention that NK cells play no significant antiviral role in LCMV infections. Data supporting this proposal were drawn from experiments of: i) acute infection with two different strains of LCMV (Tables 1 and 4); ii) persistent LCMV infection (Table 2); and iii) passive immunization studies in which no role for NK cell-mediated ADCC could be shown (Tables 4, 5 and 6).

CHAPTER IV
SPECIFICITY OF ADOPTIVE IMMUNIZATION

The process of adoptive immunization has been successfully employed to demonstrate the antiviral potential of virus-immune spleen T cells in vivo (see Chapter I). The actual mechanism by which immune T cells mediate this protection, however, is uncertain. There are two prominent hypotheses to account for this phenomenon. First, virus-specific CTL may lyse virus-infected target cells in vivo in a manner analogous to that seen in vitro. Secondly, MHC class I-restricted virus-specific T cells may release soluble factors with potential antiviral activity following their recognition of appropriate antigens. Either mechanism could adequately explain the currently available data on antiviral adoptive immunization. The experiments described below were performed to distinguish which of these two possibilities is most likely responsible for the in vivo antiviral effects of transferred immune T cells previously described.

A. Adoptive immunization by alloimmune spleen cells

Alloimmune spleen cells were generated by injecting BALB/cJ (H-2^d) lymph node cells into irradiated C3H/St (H-2^k) mice as described in Materials and Methods. The in vitro cytotoxic specificity of these cells for H-2^k target cells is shown in

Table 7. The slightly elevated lysis of "uninfected" L929 cells by LCMV-immune C3H/St splenocytes may have been due to infection of some of the L929 cells with LCMV derived from the spleen effector cells during the relatively long assay period.

Alloimmune (H-2^d anti-H-2^k) cells were then used to adoptively immunize LCMV-infected C3H/St mice. The rationale for this experiment was that, in the environment of an H-2^k host, these splenocytes would be stimulated by alloantigen to release soluble factors (eg, immune interferon) which might have an antiviral effect on LCMV replication. As shown in Table 8, however, no antiviral effect of adoptively transferred alloimmune spleen cells was seen. In comparison, mice receiving syngeneic LCMV-immune cells had significantly ($p < .001$) reduced LCMV spleen titers. Because of the short duration of the experiment (1 day), it could not be documented that the transferred alloimmune cells were actually functioning in vivo in the induction of a graft-vs-host type reaction. Additionally, these intravenously injected splenocytes may have been trapped in the lungs of the recipient mice, and an antiviral effect mediated by the cells might not have been evident in the spleen. Another system that would provide an internal control for the in vivo activity of the adoptively transferred cells was developed.

B. Adoptive immunization of dually virus-infected mice

1. LCMV and vaccinia virus. The rationale for the following

Table 7

In Vitro Cytotoxic Specificity of Alloimmune Spleen Cells

<u>Effector Cells</u> ^a	<u>% Specific Lysis</u> ^b		
	<u>Vero</u>	<u>P815 (H-2^d)</u>	<u>L-929 (H-2^k)</u>
Alloimmune (H-2 ^d anti-H-2 ^k)	-1.7	-0.4	89.
Normal C3H/St (H-2 ^k)	2.9	-0.3	0.6
LCMV-Immune C3H/St	3.9	5.4	19.

^aAlloimmune effector cells were prepared as described in Materials and Methods. LCMV-immune cells were obtained from 9 week old mice at day 8 p.i. with 8×10^4 PFU of LCMV-ARM.

^bCytotoxicity assays were run for 16 hr at E:T=6.1.

Table 8
 Adoptive Immunization of LCMV-Infected
 C3H/St Mice with Alloimmune Spleen Cells

Spleen Cells Transferred ^a	LCMV Titer Log ₁₀ PFU/Spleen ^b
None	5.0 ± 0.1
Normal C3H/St	4.8 ± 0.3
LCMV-immune C3H/St	1.5 ± 0.5
Alloimmune (H-2 ^d anti-H-2 ^k)	5.4 ± 0.15

^aRecipient mice (n=3 per group) were infected with 8×10^4 PFU LCMV-ARM i.p. 1 day before receiving 3×10^7 cells i.v. Alloimmune cells were prepared as described in Materials and Methods; LCMV-immune cells were from mice infected 8 days previously.

^bSpleens of recipient mice were removed 1 day after adoptive immunization.

series of experiments was similar to that described for the alloimmune adoptive transfer studies above. Here, mice were simultaneously infected with two different viruses (dually infected) and then adoptively immunized with histocompatible spleen cells immune to one of the two viruses. If protection was mediated by nonspecific antiviral soluble factors, the titers of both viruses might be expected to be reduced. Alternatively, if protection was mediated by a specific mechanism such as direct cytotoxicity, only the virus to which the spleen cells were immune would be expected to be reduced in titer.

The first pair of viruses used in these studies was vaccinia virus and LCMV (Armstrong strain). A plaque assay protocol was developed to allow the differential titration of each virus in the presence of the other (see Chapter II). This involved preparing two parallel plaque assays for each tissue titrated. The first of these was incubated for two days to allow for the formation of vaccinia virus plaques and then stained. No detectable LCMV plaques were present at this time. The second set of plates, containing N-methyl-isatin beta-thiosemicarbazone (MIBT) in the agarose overlay to inhibit vaccinia virus replication, was incubated for four days and then stained for LCMV plaques. The effects of MIBT on the formation of vaccinia virus and LCMV plaques are shown in Table 9. In subsequent studies (not shown), it was found to be necessary to add an additional 5 ug/ml MIBT to the LCMV plaque assay plates at day 2

Table 9
 Effect of N-Methyl-Isatin Beta-Thiosemicarbazone (MIBT)
 and Dimethyl Sulfoxide (DMSO) on Plaque Formation
 by Vaccinia Virus and LCMV on Vero Cells

Virus	Overlay Additives ^a	Virus Titer (Log ₁₀ PFU/ml) ^b
LCMV	None	4.6
	DMSO:0.1%	4.7
	MIBT:0.2 ug/ml	4.6
	MIBT:1.0 ug/ml	4.6
	MIBT:5.0 ug/ml	4.2
Vaccinia	None	5.2
	DMSO:0.1%	5.1
	MIBT:0.2 ug/ml	5.0
	MIBT:1.0 ug/ml	4.4
	MIBT:5.0 ug/ml	< 1.0

^aAdditive concentration in agarose overlay.

^bLCMV plaques were stained after 4 days incubation and
 vaccinia virus plaques after 2 days.

of incubation to completely suppress vaccinia virus replication for the duration of the incubation period. This additional MIBT had no apparent effect on LCMV titers.

Vaccinia virus-immune spleen cells were prepared at day 7 p.i. and tested for their cytotoxic activity in vitro. Table 10 demonstrates that these effector cells preferentially lysed histocompatible target cells infected with vaccinia virus.

These effector cells were then used to adoptively immunize mice infected with vaccinia virus alone, or mice dually infected with vaccinia virus and LCMV. In Table 11 (Expt. 1), vaccinia virus-immune spleen cells failed to successfully immunize either singly or dually infected mice to vaccinia virus at day 1 after cell transfer ($p > .05$ vs recipients of normal cells). Waiting 2 days post-transfer of the immune cells before harvesting organs (Expt. 2) yielded a reduction in titer that was statistically significant ($p < .001$). Vaccinia virus titers among individual mice exhibited less variation at this time.

These experiments were repeated with the transfer of LCMV-immune or vaccinia virus-immune spleen cells into dually infected recipients (Table 12). In Expt. 1, LCMV-immune splenocytes protected mice against LCMV ($p < .001$) without significantly reducing vaccinia virus titers ($p > .05$). Similar results were seen in Expt. 2 where LCMV-immune cells again significantly reduced LCMV titers ($p < .01$) but not vaccinia virus titers ($p > .05$). Vaccinia virus-immune spleen cells were successful in

Table 10

Generation of Vaccinia Virus-Immune Spleen Cells

<u>Expt</u>	<u>Effector Cells</u> ^a	<u>% Specific Lysis</u> ^b			
		<u>L-929</u>		<u>L-929 + Vaccinia</u>	
		<u>100:1</u>	<u>50:1</u>	<u>100:1</u>	<u>50:1</u>
1	Normal	1.2	-1.0	3.5	2.0
	Vaccinia Immune	-0.5	2.5	28.	21.
2	Vaccinia Immune	-1.2	1.4	49.	32.

^aSix week old C3H/St mice were untreated (normal) or infected i.p. with 1×10^7 PFU vaccinia virus 7 days earlier (vaccinia immune).

^b ^{51}Cr release assays with pooled spleen effector cells (n=4-6 mice per group) were run for 6 hr.

Table 11

Adoptive Immunization by Vaccinia Virus-Immune Spleen Cells

Expt	Virus Inoculated ^a	Spleen Cells Transferred ^b	Virus Titer ^c	
			Log ₁₀ PFU/Spleen	
			Vaccinia	LCMV
1	Vaccinia	Normal	4.6 ± 0.2	-
		Vaccinia Immune	3.7 ± 1.5	-
	Vaccinia + LCMV	Normal	4.4 ± 0.7	5.7 ± 0.2
		Vaccinia Immune	5.0 ± 0.2	5.9 ± 0.3
2	Vaccinia	Normal	3.8 ± 0.2	-
		Vaccinia Immune	2.9 ± 0.1	-

^aC3H/St mice (8 week old; n=4 per group) were infected i.p. with 1×10^7 PFU vaccinia virus alone, or with vaccinia virus and 8×10^4 PFU LCMV 24 hr before cell transfer.

^bSpleen cells (3×10^7) were from normal or vaccinia virus-infected (day 7 p.i.) C3H/St donors.

^cRecipient spleens were titrated at 1 (Exp 1) or 2 (Exp 2) days after cell transfer.

Table 12

Adoptive Immunization by Immune Spleen Cells:

Vaccinia Plus LCMV Dually Infected Mice

Exp	Virus Inoculated ^a	Spleen Cells Transferred ^b	Virus Titer ^c	
			Log ₁₀ PFU/Spleen	
			Vaccinia	LCMV
1	Vaccinia + LCMV	Normal	3.9 ± 0.4	6.0 ± 0.2
		LCMV Immune	3.4 ± 0.7	1.5 ± 0.5
2	Vaccinia + LCMV	Normal	5.3 ± 0.3	6.1 ± 0.1
		LCMV Immune	4.8 ± 0.4	4.4 ± 1.1
		Vaccinia Immune	4.5 ± 0.4	5.9 ± 0.3

^aC3H/St mice (n=4-5 per group) were infected i.p. with 1×10^7 PFU vaccinia virus and 8×10^4 LCMV 1 day before cell transfer.

^bSpleen cells (3×10^7) were from normal or day 7 p.i. LCMV- or vaccinia virus-immune C3H/St donors.

^cSpleens of recipient mice were titrated for virus 2 days after cell transfer.

adoptively immunizing mice to vaccinia virus ($p < .01$) but not to LCMV. These data are consistent, therefore, with the hypothesis that adoptive immunization by virus-immune spleen cells is mediated in a highly specific manner (e.g., direct cytotoxicity) rather than through the release of non-specific antiviral factors.

2. LCMV and Pichinde virus. The experiments of adoptive immunization to vaccinia virus (Tables 11 and 12) often led to small, and sometimes statistically insignificant, reductions in vaccinia virus titers. Another dual-virus system was thus established, this time using two closely related arenaviruses, LCMV and Pichinde virus (PV).

A plaque assay for the differential titration of LCMV and PV in dually infected spleens was designed as outlined in Chapter II. Briefly, PV was specifically titrated by neutralizing LCMV with LCMV-immune guinea pig serum. LCMV was titrated by staining plaque assay plates with INT which stains uninfected and PV-infected cells but not LCMV-infected cells (Logan et al., 1975). Thus, LCMV plaques appear clear on a stained background. Table 13 shows that treatment with GP anti-LCMV serum did not inhibit PV infectivity and that INT stain was ineffective in demonstrating PV plaques.

It was also necessary to demonstrate that virus-specific CTL generated during PV and LCMV infections were not cross-reactive in their lytic activity for target cells infected with the

Table 13
Differential Titration of Pichinde Virus (PV) and LCMV

Exp	Virus	GP Anti-LCMV ^a	Plaque Assay	Apparent Virus Titer
			Stain ^b	Log ₁₀ PFU/ml
1	LCMV	-	Neutral Red	4.0
		+	Neutral Red	<2.0
		-	INT	4.0
	PV	-	Neutral Red	4.5
		+	Neutral Red	4.6
		-	INT	<2.0
2	LCMV	-	INT	5.3
		+	Neutral Red	<2.0
	PV	-	INT	<2.0
		+	Neutral Red	7.0

^aAliquots of virus stocks were untreated or treated with guinea pig (GP) anti-LCMV serum before titration as described in Materials and Methods.

^bPlaque assay plates (vero cells) were stained at day 4 with neutral red dye or iodophenyl nitrophenyl phenyltetrazolium chloride (INT) as described in Materials and Methods.

heterologous arenavirus. This was shown to be the case as LCMV-immune spleen cells lysed LCMV-infected targets, and PV-immune spleen cells lysed PV-infected targets (Table 14). Neither effector cell population lysed targets infected with the heterologous virus to a significant degree, in agreement with previous studies (Buchmeier et al., 1980).

Spleen cells from PV-infected mice were harvested at days 6-9 p.i. and tested for their ability to adoptively immunize recipient mice acutely infected with PV. The data in Table 15 demonstrate that all immune effector cell populations were approximately equivalent in their ability to successfully transfer immunity to PV compared to normal spleen cells ($p < .05$ - $p < .02$). PV-immune cells obtained at day 7 p.i. were chosen for the remaining experiments. Additionally, two days were allowed to elapse following cell transfer in the hope of achieving a greater reduction in virus titer than that seen earlier (Table 15) where spleens were harvested 1 day after transfer.

LCMV-immune or PV-immune spleen cells were transferred to recipient mice acutely infected with either LCMV, PV, or both viruses. Table 16 (Expt. 1) shows the results of transferring LCMV-immune spleen cells to singly and dually infected mice. Compared to normal spleen cells, LCMV-immune spleen cells significantly ($p < .001$) reduced LCMV titers in mice infected with LCMV alone, but not in mice infected with PV alone. In mice infected with both viruses, only the LCMV titers were reduced (p

Table 14

In Vitro Cytotoxic Specificity of Virus-Immune Spleen Cells

<u>Spleen Cells</u> ^a	<u>% Specific Lysis</u> ^b		
	<u>L-292</u>	<u>L-929 + PV</u>	<u>L-929 + LCMV</u>
Normal C3H	-1.0	0.3	0.2
LCMV-Immune	3.4	0.7	32.
PV-Immune	1.1	33.	4.1

^aSpleen cells were from normal or day 7 virus-immune C3H/St mice.

^bCytotoxicity assays were run for 6 hr at E:T=50:1.

Table 15

Adoptive Immunization by PV-Immune

Spleen Cells Prepared at Different Times Postinfection

Spleen Cells Transferred ^a	Virus Titer Log ₁₀ PFU/Spleen ^b
Normal	5.3 ± 0.3
Day 6 Immune	4.5 ± 0.1
Day 7 Immune	4.7 ± 0.2
Day 8 Immune	4.5 ± 0.1
Day 9 Immune	4.7 ± 0.2

^aSpleen cells were from normal or PV-infected (1×10^6 PFU i.p.) C3H/St mice at various days p.i. Cell transfers (3×10^7 i.v.) were all performed on the same day into C3H/St mice infected i.p. 1 day earlier with PV.

^bSpleens of recipient mice (n=3 per group) were titrated 2 days after cell transfer.

Table 16
 Specificity of Arenavirus-Immune
 Spleen Cells in Adoptively Immunized Mice

Expt	Virus Inoculated ^a	Spleen Cells Transferred ^b	Virus Titer ^c	
			Log ₁₀ PFU/Spleen	
			PV	LCMV
1	LCMV	Normal	-	4.9 ± .38
		LCMV-Immune	-	2.4 ± .10
	PV	Normal	5.2 ± .05	-
		LCMV-Immune	5.4 ± .08	-
	LCMV + PV	Normal	5.5 ± .05	4.1 ± .82
		LCMV-Immune	5.5 ± .18	2.8 ± .18
2	LCMV	Normal	-	4.4 ± .42
		PV-Immune	-	4.6 ± .49
	PV	Normal	4.4 ± .18	-
		PV-Immune	3.2 ± .76	-
	LCMV + PV	Normal	4.6 ± .13	3.7 ± .44
		PV-Immune	3.0 ± .11	3.5 ± .21

Table 16 (cont'd)

^aC3H/St mice (n=4 per group) were infected with LCMV (8×10^4 PFU i.p.), PV (1×10^6 PFU i.v.) or both viruses 24 hr before cell transfer.

^bMice received 5 (Expt. 1) or 8 (Expt. 2) $\times 10^7$ normal or day 7 virus-immune C3H/St spleen cells i.v.

^cSpleens of recipient mice were removed and titrated for virus 2 days after cell transfer.

<.05) while PV titers were unaffected. In the reciprocal experiment (Table 16, Expt. 2), PV-immune spleen cells were adoptively transferred. These cells had no effect on LCMV titers in mice singly infected with LCMV or in dually infected mice. However, PV titers were significantly reduced in mice singly infected with PV ($p < .05$) or dually infected with PV and LCMV ($p < .001$). In these experiments, therefore, the specificity of adoptive immunization against arenavirus infection in vivo (Table 16) correlated with the specificity of virus-immune CTL recognition patterns in vitro (Table 14).

To examine the possibility that the fine specificity of the immunizing cells was due to low titers of the two viruses growing in widely separated regions of the spleen at the time of adoptive transfer (day 1 p.i.), recipient mice were lethally irradiated (1000 R, gamma irradiation) to suppress their own immune response, which would allow for a longer time of infection, and then dually infected with LCMV and PV. These mice were adoptively immunized with LCMV-immune cells on day 3 p.i. and spleen virus titers were determined 2 days after cell transfer. The data in Table 17 again demonstrate the specific reduction of LCMV titers in the spleen following LCMV-immune cell transfer with no effect observed on the heterologous PV titers.

3. Localization of LCMV and PV antigens in dually infected spleens. In a parallel experiment, the spleens of lethally irradiated, dually infected mice were obtained 3 days p.i. and

Table 17

Adoptive Immunization of Lethally Irradiated, Dually Infected Mice^a

Spleen Cells Transferred ^b	Virus Titer ^c	
	Log ₁₀ PFU/Spleen	
	PV	LCMV
Normal	3.4 ± .15	4.0 ± .50
LCMV-Immune	3.4 ± .15	2.7 ± .32*

^aRecipient C3H/St mice (n=4 per group) were irradiated (1000 R gamma irradiation) and then infected with 8×10^4 PFU LCMV i.p. and 1×10^6 PFU PV i.p.

^bOn day 3 postinfection, mice received 5×10^7 normal or day 7 LCMV-immune C3H/St spleen cells i.v.

^cSpleens of recipient mice were removed 2 days after cell transfer for titration of virus.

processed for virus-specific immunofluorescent staining. Serial four micron frozen sections were stained with LCMV- or PV-immune guinea pig sera. Side-by-side comparison of these sections (Figure 1) indicated that both viruses were indeed growing in very close proximity to one another at the time of adoptive immunization.

Thus, under the present conditions where transferred virus-immune splenocytes were stimulated by antigen to which they had been primed, no non-specific antiviral effect was evident. These results support the concept that T cell-dependent adoptive immunization involves an extremely localized antiviral effect by the immune splenocytes, and the data are consistent with a mode of action involving direct cytotoxicity.

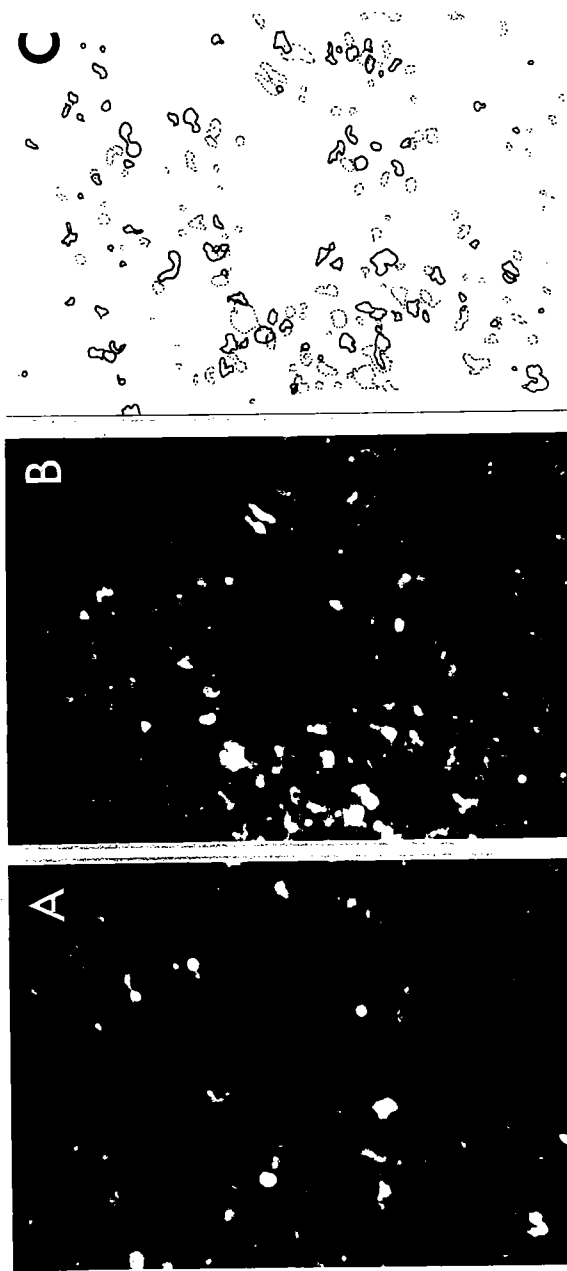


Figure 1

Figure 1. Localization of LCMV and PV antigens in spleens of dually infected mice. Four micron serial sections of dually infected spleens were incubated with LCMV- or PV-immune guinea pig serum, washed, and stained with rhodamine-conjugated goat anti-guinea pig serum. Neither virus-specific antiserum stained uninfected spleens or spleens singly infected with the heterologous virus (not shown). Panel A (LCMV antigen) and panel B (PV antigen) represent the same area of adjacent sections. Panel C is a composite drawing (LCMV antigen, solid lines; PV antigen, dotted lines) made by superimposing panels A and B. Original magnification X 400.

CHAPTER V

CELL-MEDIATED IMMUNITY IN VIRAL HEPATITIS

The liver is a major organ for the replication of many viruses and the sole organ for the replication of some. Virus-induced hepatitis is frequently a severe life-threatening disease affecting millions of people worldwide, but little is known about the cytotoxic inflammatory cell population within the diseased liver. The following section of the thesis analyzes the nature of the inflammatory cell infiltrate in the liver during the course of viral hepatitis induced by infection with several viral agents.

A. Effect of perfusate enzyme concentration on leukocyte yields from perfused livers

In preliminary experiments (not shown), a variety of different enzymes were tested for their ability to aid in the dissociation of the liver into a single cell suspension following in situ perfusion. The enzymes examined individually and in various combinations included collagenase (types I and IV), pronase, hyaluronidase, and dispase (neutral protease). A combination of collagenase (type I) and dispase appeared to be promising in initial trials and was examined more closely, varying the concentrations of the two enzymes (Table 18). Since it was deemed desirable to minimize potential proteolytic damage

Table 18

In Situ Liver Perfusion: Effect of Enzyme
Concentration on Leukocyte Yield^a

<u>Enzyme Concentration (%)</u>		<u>Total Leukocytes</u>
<u>Collagenase</u>	<u>Dispase</u>	<u>per Liver (X10⁻⁶)</u>
.05	.10	9.6
.05	.05	10.
.025	.025	3.0

^aLivers of C57BL/6J mice (6 week old) were perfused as described in Materials and Methods, except the enzyme concentrations listed were employed.

to leukocyte cell surface proteins and receptors while maximizing cell yields, the intermediate enzyme concentrations of .05% collagenase and .05% dispase (Table 18) were used in all subsequent perfusion studies.

B. Effect of enzymes on spleen NK cell activity

Since enzyme perfusion was used to isolate liver leukocytes, it was not possible to determine the effects (if any) of in situ enzyme exposure on these cells. The enzymes were tested for their effect on NK cell lysis mediated by spleen cells. Spleen cells were exposed to collagenase and dispase in vitro for a time period equivalent to the period of exposure of liver leukocytes to enzyme in situ. Table 19 shows that exposure of spleen cells to enzyme had no diminishing effect on their NK cell activity. Rather, enzyme exposure may actually have had a slight enhancing effect on lysis. However, since liver leukocytes from all mice would be exposed to the same enzymes during in situ perfusion, this minor effect on lytic activity would likely be uniform and was not taken into account in further determinations of cytotoxic activity.

C. Comparison of hepatotropic and non-hepatotropic LCMV infections

The induction of NK cell and CTL activities and the accumulation of LGL in the liver were studied on days 1-14 p.i.

Table 19

Effect of Enzyme Treatment on Spleen NK Cell Activity^a

<u>Enzyme Concentration (%)</u>		<u>% Specific Lysis</u> ^b	
<u>Collagenase</u>	<u>Dispase</u>	<u>YAC-1</u>	<u>L-929</u>
0	0	13.	2.1
.05	.10	16.	3.1
.05	.05	18.	3.1
.025	.025	15.	1.7

^aAliquots of endogenous (normal) C57BL/6J spleen cells (3.5×10^7) were incubated for 15 min at room temperature in HBSS containing the indicated concentrations of enzymes. Deoxyribonuclease was added (100 U/ml final concentration) and incubation continued for an additional 10 min. The cells were washed and resuspended for use in ⁵¹Cr release assays.

^bCytotoxicity assays were run for 4 hr at E:T=100:1.

in animals infected i.p. with either LCMV-ARM or LCMV-WE. LCMV-ARM is generally non-hepatotropic and grows poorly in the liver during acute infection, whereas the LCMV-WE strain is hepatotropic and replicates in the liver to high titer (Welsh, 1984b). Both viruses induced an increase in NK cell activity detected against both YAC-1 and L929 cells by day 1 p.i. that peaked at days 3-5 p.i. and declined thereafter (Figure 2D and 2E). The appearance and progressive increase of NK cell activity through day 5 p.i. were paralleled by a progressive increase in LGL number beginning on day 1 p.i. (Figure 2B). Virus-specific CTL activity began to increase on day 5 p.i. with both viruses and peaked on day 7 p.i. (Figure 2F). By day 7 p.i., the LCMV-WE infection induced an average 28-fold increase in the number of liver leukocytes, while the LCMV-ARM infection induced only a 5-fold increase (Figure 2A). These differences in total leukocyte number were confirmed histologically: LCMV-WE-infected livers had markedly greater infiltrations of mononuclear inflammatory cells than LCMV-ARM-infected livers (Figure 3). The centers of focal accumulations of mononuclear cells occasionally contained one or two necrotic hepatocytes with pyknotic nuclei and intensely eosinophilic cytoplasm. Because LCMV is relatively non-cytopathic (Welsh and Pfau, 1972), it is likely that hepatocyte death results from an immunopathological mechanism (see Chapter I). When the total lytic capacities of the liver leukocyte populations were calculated, the LCMV-WE infection

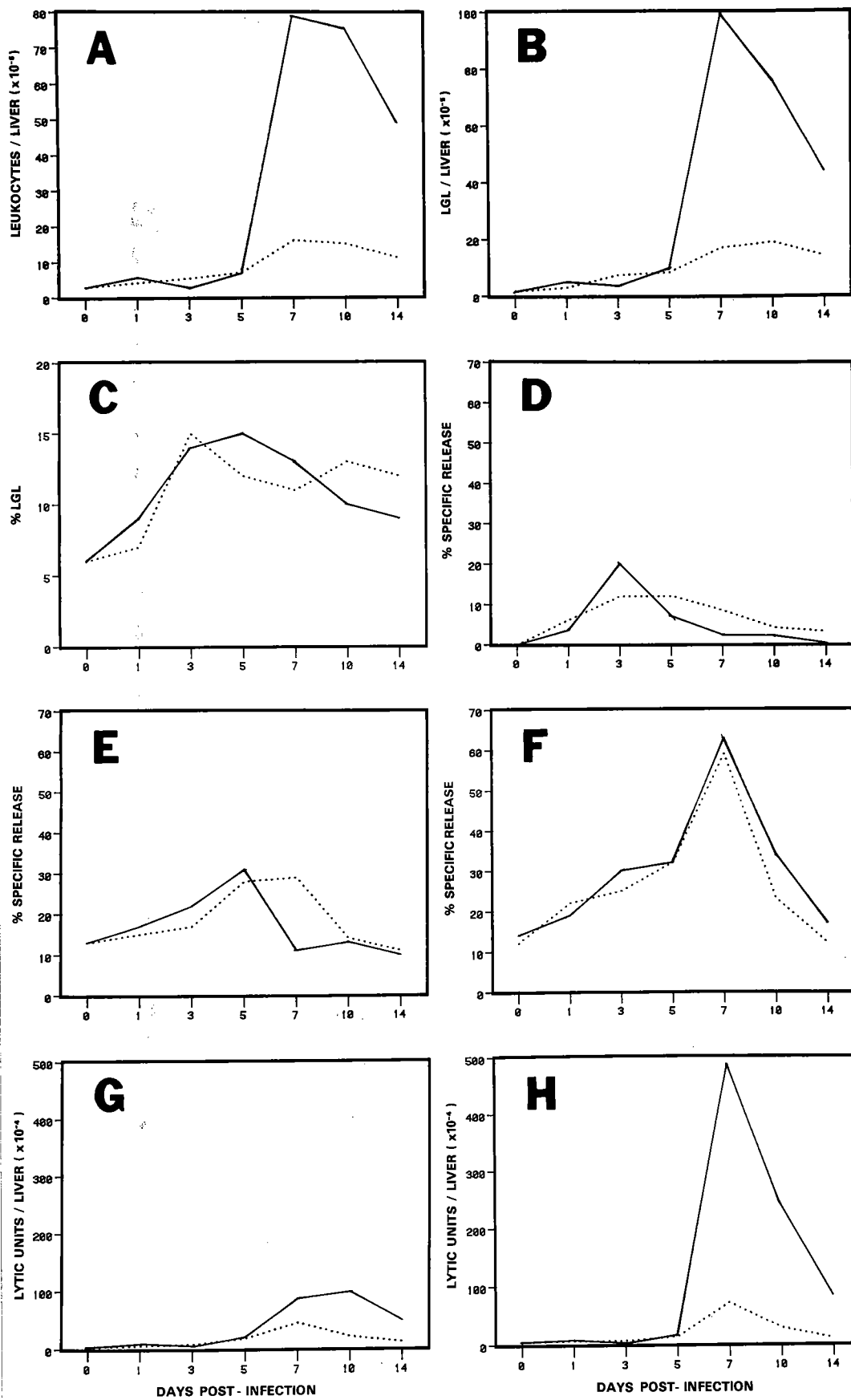


Figure 2

Figure 2. Kinetics of the appearance of LGL and of NK cell and CTL cytotoxic activities in the livers of C3H/St mice infected with LCMV-ARM (dotted line) or LCMV-WE (solid line). A. Total leukocytes per liver. B. Total LGL per liver. C. Percent LGL in the leukocyte population. D. Percent specific ^{51}Cr release from L929 targets at E:T=10 in 12 hr assay. E. Percent specific ^{51}Cr release from YAC-1 targets at E:T=10 in 4 hr assay. F. Percent specific ^{51}Cr release from LCMV-infected L929 cells at E:T=10 in 12 hr assay. G. NK cell lytic units per liver (calculated from % YAC-1 lysis as described in Materials and Methods). H. CTL lytic units per liver (calculated from % virus-specific lysis as described in Materials and Methods).

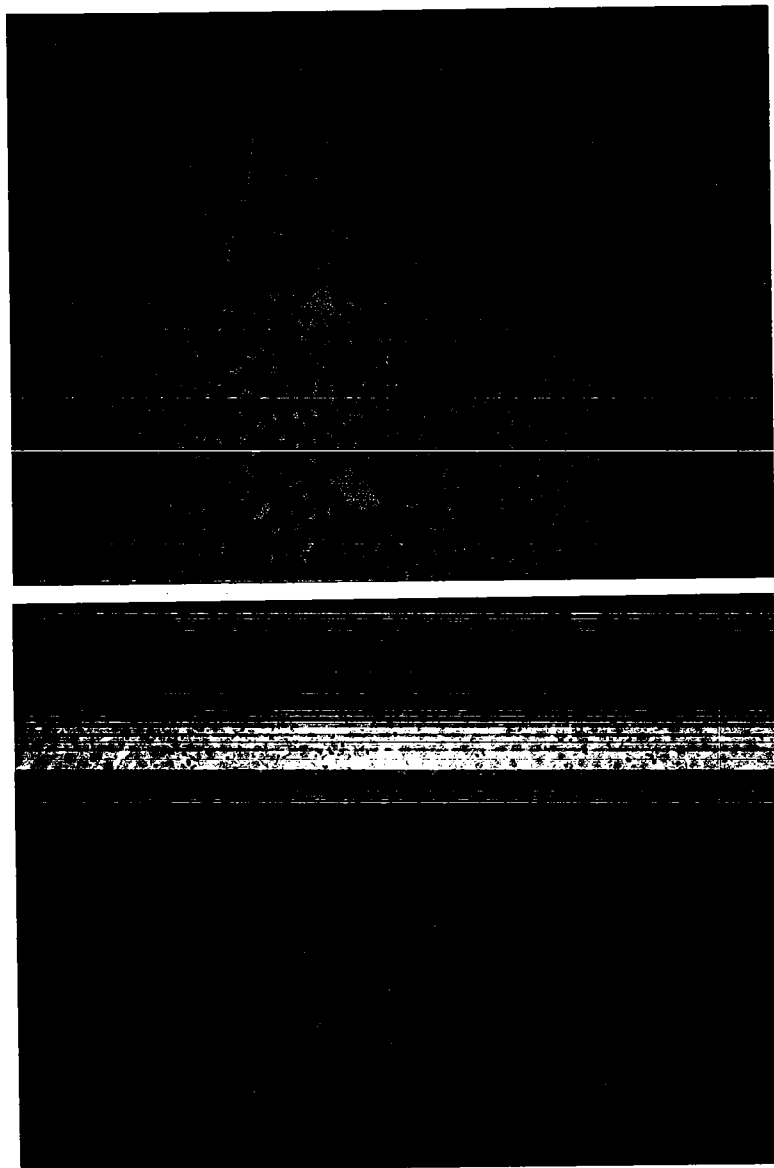


Figure 3

Figure 3. Day 7 p.i. liver histology of C3H/St mice infected with LCMV-ARM (Panel A) or LCMV-WE (Panel B). Livers of LCMV-infected mice were fixed by perfusion with 10% formalin at day 7 p.i. Note the greater numbers of mononuclear inflammatory cells in the parenchyma of the LCMV-WE-infected liver. Hematoxylin and eosin, X 100.

resulted in 10-fold higher total CTL lytic units on day 7 p.i. than did LCMV-ARM infection (Figure 2H). The levels of day 7 CTL activity closely correlated with the total numbers of LGL isolated on day 7 from the livers of mice infected with LCMV-WE or LCMV-ARM (Figure 2B).

D. Characterization of day 3 and day 7 cytotoxic effector cells

The patterns of cytotoxic activities present at days 3 and 7 p.i. (Figure 2) were suggestive of the NK cell and CTL lytic profiles reported previously in the spleen (Welsh, 1978). To further characterize these effector cell populations, liver leukocytes were treated with various antibodies and complement (C) in vitro. The cells remaining after treatment were tested for cytotoxic activity and were examined for the presence of LGL on stained cytocentrifuge smears. The data in Table 20 (Expt. 1) indicate that day 3 cytotoxic activity against YAC-1 cells was completely sensitive to treatment with anti-asialo GM₁ + C and slightly reduced by anti-Thy 1.2 + C. Treatment with C and J11d antibody (recognizing a marker on granulocytes and B cells (Bruce et al., 1981)) had no effect on lysis. Changes in the number of cells morphologically identified as LGL paralleled the changes in lytic activity: anti-asialo GM₁ + C treatment markedly reduced the number of LGL, anti-Thy 1.2 + C resulted in a partial depletion (64%), and J11d + C was without effect on LGL number. Thus, the phenotype for the day 3 p.i. effector cells is

Table 20

Characterization of Liver Leukocytes Isolated at Days 3 and 7 P.I. with LCMV-ARM^a

Expt.	Treatment	% Specific		LGL x10 ⁵ (% Reduction)
		Lysis at E:T ^b		
		20:1	7:1	
1. Day 3 effectors/YAC-1 targets	GpC control	29.	12.	1.4
	Anti-asialo GM1 + GpC	1.0	0.2	0.1 (93)
	Anti-Thy-1.2 + GpC	22.	10.	0.5 (64)
	RbC control	35.	15.	1.0
	J11d + RbC	34.	15.	1.1 (-10)
2. Day 7 effectors/% virus-specific lysis	GpC control	<u>7:1</u>	<u>2:1</u>	
	GpC control	30.	17.	15.
	Anti-Thy-1.2 + GpC	8.8	0.7	4.5 (70)
	RbC control	32.	15.	8.4
	Anti-Lyt-2 + RbC	12.	2.3	5.5 (35)

^aLiver leukocytes isolated from C3H/St mice at day 3 p.i. (Expt. 1) or day 7 p.i. (Expt. 2) with LCMV-ARM were treated with antibody and/or C as described in Materials and Methods.

^bCytotoxicity assays were run for 4 hr (Expt. 1) or 12 hr (Expt. 2).

consistent with the reported phenotype of NK cells (Biron, 1986): asialo GM_1^+ , Thy 1.2 $^{+/-}$, J11d $^-$, YAC-1 killer, and LGL morphology.

Liver leukocytes isolated at day 7 p.i. were treated with antibody reagents known to react with determinants present on CTL. As shown in Table 20 (Expt. 2), treatments with either anti-Thy 1.2 + C or anti-Lyt 2 + C significantly reduced the virus-specific lysis of infected target cells. These treatments also resulted in the depletion of a significant proportion of the LGL from the effector cell population.

To further confirm the surface phenotype and LGL morphology of effector cells obtained at days 3 and 7 p.i. with LCMV, liver leukocytes were examined by FACS analysis following staining with monoclonal anti-Lyt-2 antibody. Eleven percent of the day 3 p.i. liver leukocyte population was Lyt-2 $^+$ (Table 21). The vast majority (89%) of day 3 p.i. leukocytes were Lyt-2 $^-$, and 13% of these cells were LGL. The Lyt-2 $^-$ LGL accounted for 77% of all LGL present at day 3 p.i., and this population was enriched for NK cell activity. No virus-specific lytic activity was detectable at this time p.i. in any population.

In contrast to day 3 p.i. leukocytes, twenty-seven percent of the day 7 p.i. liver leukocyte population was Lyt-2 $^+$. FACS-sorted Lyt-2 $^+$ cells were enriched at least two-fold for virus-specific lytic activity compared to stained but unseparated cells, while NK cell activity was reduced (Table 21).

Table 21
 Lyt-2 Phenotype of LGL at Days 3 and 7 p.i. with LCMV-ARM^a

Cell Population	% LGL	% Virus-Specific Lysis ^b	% YAC-1 Lysis ^b
Day 3 Lyt-2 ⁺ (11)	34 (23)	< 1	11
Lyt-2 ⁻ (89)	13 (77)	< 1	20
Unsorted	20	< 1	16
Day 7 Lyt-2 ⁺ (27)	42 (58)	57	3.1
Lyt-2 ⁻ (73)	13 (42)	10	16.
Unsorted	23	27	6.9

^aLiver leukocytes from C3H/St mice at day 7 p.i. with LCMV-ARM were stained with monoclonal anti-Lyt-2 antibody and sorted into Lyt-2⁺ and Lyt-2⁻ populations. Values in parentheses indicate the percentage of all cells of a particular type expressing the indicated Lyt-2 phenotype.

^bNK cell assays against YAC-1 targets were run for 4 hr at E/T of 3:1, and CTL assays against uninfected and LCMV-infected L-929 cells were run for 14 hr at E/T of 3:1.

Conversely, while Lyt-2⁻ cells were depleted of virus-specific cytotoxic effectors, they were enriched for cells expressing NK cell activity. Lyt-2⁺ cells were enriched almost two-fold for cells possessing LGL morphology, and they accounted for 54% of all LGL present. Day 7 p.i. Lyt-2⁻ cells were LGL-depleted compared to unfractionated cells (Table 21). Thus, CTL activity and cells bearing LGL morphology were found to co-purify in a population of leukocytes expressing the CTL marker Lyt-2.

E. Cytotoxicity pattern of day 7 post-LCMV infection liver leukocytes

Spleen CTL induced during LCMV infection are virus-specific and H-2-restricted effector cells (Zinkernagel and Doherty, 1975). When tested for lysis against various targets, liver effector cells isolated from C3H/St mice at day 7 p.i. had typical CTL killing patterns (Table 22). These effectors strongly lysed LCMV-infected histocompatible cells (L929 + LCMV) but not LCMV-infected histoincompatible cells (MC57G + LCMV). They also exhibited minimal lysis against histocompatible cells either uninfected or infected with Pichinde virus (L929 + PV). Earlier experiments (Table 14) showed that PV-infected cells were highly sensitive targets for PV-immune CTL. These experiments confirm that the effector cells isolated from liver at day 7 p.i. with LCMV contain classical virus-specific CTL.

Table 22
 Cytotoxicity Pattern of Day 7 p.i.
 Effector Cells From LCMV-ARM-Infected Liver^a

E:T Ratio	% Specific Lysis			
	L-929	L-929 + LCMV	L-929 + PV	MC57G + LCMV
20	13	59	16	18
7	4.3	37	5.0	4.7
2	2.4	20	-0.6	4.7

^aLiver leukocytes from C3H/St mice (H-2^k) at day 7 p.i. with LCMV-ARM were tested for lytic activity against uninfected L-929 cells (H-2^k), L-929 cells infected with LCMV (L-929 + LCMV) or Pichinde virus (L-929 + PV), and MC57G cells (H-2^b) infected with LCMV (MC57G + LCMV) in 12 hr ⁵¹Cr-release assays.

F. Adherence properties of liver NK/LGL

Liver leukocytes were isolated from control and poly I:C-treated mice. Plastic-adherent cells were removed by incubation in plastic petri dishes. NK cell lytic activity for YAC-1 targets was enriched in non-adherent populations from both control and poly I:C-activated liver leukocytes (Table 23). While cells with LGL morphology were enriched in the non-adherent leukocyte fraction from poly I:C-treated mice, a similar enrichment was not observed for control liver leukocytes. The reason for this apparent difference in adherence between control and activated NK/LGL may be related to a lesser depletion of macrophages and non-granular lymphocytes from the control leukocytes than from the poly I:C-treated leukocytes (data not shown), resulting in no overall enrichment of LGL in the non-adherent control population.

G. Depletion of LGL in vivo

Mice were treated in vivo with anti-asialo GM₁ at the time of viral infection, and liver leukocytes and splenocytes were isolated at day 3 p.i. Table 24 (Expt. 1) demonstrates that in vivo anti-asialo GM₁ serum treatment was highly effective ($p < .01$) at depleting NK cell activity (YAC-1 lysis) in both the liver and spleen during virus infection. This treatment also significantly reduced both the percentage ($p < .001$) and absolute number of LGL ($p < .001$) in the liver leukocyte population without

Table 23
Adherence Properties of Liver-Derived NK/LGL

<u>Liver Leukocytes^a</u>	<u>Treatment^b</u>	<u>% LGL</u>	<u>% YAC-1 Lysis^c</u>
Control	Unseparated	7	17.
	Non-adherent	6	25.
Poly I:C	Unseparated	15	62.
	Non-adherent	25	75.

^aLiver leukocytes were pooled from control or day 2 poly I:C-injected (100 ug i.p.) C57BL/6J mice.

^bNon-adherent cells were prepared as described in Materials and Methods.

^c4 hr assays, E:T=33:1.

Table 24
Effects of Anti-Asialo GM1 Treatment In Vivo
on Accumulation of NK/LGL and CTL/LGL in Liver During ICMV Hepatitis^a

Expt.	Anti-Asialo GM1	% YAC-1 Lysis ^b		Total Leukocytes per Liver (x 10 ⁻⁶)	% LGL	Total IGL per Liver (x 10 ⁻⁵)	% Virus-Specific Lysis ^c	
		Liver	Spleen				Liver	Spleen
1. Day 3	-	43 ± 11	36 ± 8.3	1.7 ± 0.1	25 ± 0.3	4.4 ± 0.3	ND	ND
	+	1.6 ± 0.6	4.3 ± 0.8	2.0 ± 0.6	5 ± 1.0	1.0 ± 0.3	ND	ND
2. Day 7	-	15 ± 3.1	11 ± 3.8	60 ± 8.1	14 ± 3.8	86 ± 24	41 ± 4.4	38 ± 10
	+	6.3 ± 0.6	4.3 ± 0.1	59 ± 3.0	12 ± 2.0	71 ± 16	50 ± 6.1	40 ± 7

^aSplenocytes and liver leukocytes were obtained from C3H/St mice (n=3 per group) at day 3 p.i. (Expt. 1) and at day 7 p.i. (Expt. 2) with ICMV-WE. Antiserum-treated mice were injected i.v. with 0.2 ml anti-asialo GM1 (1:10 dilution) at the time of infection (day 0; Expt. 1) or at days 0 and 4 p.i. (Expt. 2).

^bYAC-1 assays were run for 4 hr at liver leukocyte E:T=30 and spleen E:T=100.

^cPercent virus-specific lysis was determined in 16 hr ⁵¹Cr-release assays at liver leukocyte E:T=3 and spleen E:T=11.

reducing the total number of liver leukocytes present in these animals.

The sensitivity of the generation of the day 7 effector cells was similarly tested. In contrast to the day 3 NK/LGL, the accumulation of day 7 CTL/LGL in the liver was relatively resistant to a double treatment in vivo with anti-asialo GM₁ serum (Table 24, Expt. 2). This treatment eliminated most of the relatively low level of NK-like cytotoxicity against YAC-1 cells ($p < .05$) without reducing the CTL-like cytotoxicity on LCMV-infected L929 cells.

H. LGL response in C57BL/6J mice

To be certain that the differential in the liver-associated CTL response during LCMV-WE infection vs. LCMV-ARM infection was not peculiar to the C3H/St mouse strain, C57BL/6J mice were infected with LCMV-WE or LCMV-ARM, and the CTL responses were compared. Livers of C57BL/6J mice infected with LCMV-WE had significantly higher numbers of total leukocytes ($p < .02$) and total LGL ($p < .001$) as well as 2-fold higher CTL lytic units than did those from LCMV-ARM-infected mice (Table 25). These results were similar to those observed with C3H/St mice (Figure 2). Surprising, however, was the finding that this difference in the liver CTL/LGL activity was manifested despite very low levels of CTL activity in the spleens of the LCMV-WE-infected mice (Table 25). The spleens from the LCMV-WE-infected mice were smaller and

Table 25

CTL Activity in Livers but not in Spleens of C57BL/6J Mice Infected With LCMV-ARM^a

Effectors (x10 ⁻⁶)	Total Leukocytes per		% LGL (Liver)	Total LGL per Liver (x 10 ⁻⁵)	% Virus-Specific Lysis		Total CTL Lytic Units (x 10 ⁴) ^b	
	Liver (x10 ⁻⁶)	Spleen (x10 ⁻⁷)			Liver	Spleen	Liver	Spleen
Control	10 ± 2.8	4.7 ± 1	4.0 ± 1.4	3.8 ± 0.3	-	-	-	-
LCMV-ARM	17 ± 1.4	11 ± 0.7	18 ± 5.7	30 ± 7.1	48 ± 9.9	54 ± 13	55 ± 9.9	114 ± 36
LCMV-WE	63 ± 21	3.6 ± 1	12 ± 4.2	71 ± 1.3	25 ± 0.7	13 ± 0.7	108 ± 4.1	8.8 ± 1.8

^aLiver and spleen effector cells were isolated at day 7 p.i.^bLytic unit calculations are based upon percent virus-specific lysis determinations from 5 hr ⁵¹Cr-release assays at liver leukocyte E:T=20 and spleen E:T=50.

contained 67% fewer leukocytes than spleens from LCMV-ARM-infected mice. Thus, C57BL/6J mice infected with either strain of LCMV were capable of mounting a CTL response, but that response was concentrated almost exclusively in the livers of mice infected with hepatotropic LCMV-WE, whereas the CTL response predominated in the spleens of mice infected with non-hepatotropic LCMV-ARM.

I. Thymic dependence of leukocyte accumulation

The reason for the large increase in total liver leukocytes following LCMV-WE infection as compared to LCMV-ARM infection (Figure 2, Table 25) was unclear. To determine whether specific T cell-mediated immunity played a role in this difference, T cell-deficient BALB/c nu/nu mice and immunocompetent nu/+ littermates were infected with either LCMV-ARM or LCMV-WE, and liver leukocytes were isolated at day 7 p.i. The data in Table 26 indicate that the T cell-sufficient nu/+ mice infected with LCMV-WE accumulated more liver leukocytes than did nu/+ mice infected with LCMV-ARM ($p < .01$). This appeared to be thymus- and, therefore, probably T cell-dependent, as nu/nu mice infected with either LCMV-WE or LCMV-ARM had lower numbers of liver leukocytes than nu/+ mice infected with LCMV-WE.

Table 26
Inflammatory Cell Responses in Liver
at Day 7 p.i. with LCMV-WE are Thymus Dependent^a

Mouse Strain	Virus Strain	Total Leukocytes per Liver ($\times 10^{-6}$)
nu/nu	LCMV-ARM	8.8 \pm 1.9
nu/+	LCMV-ARM	12. \pm 2.3
nu/nu	LCMV-WE	7.9 \pm 1
nu/+	LCMV-WE	37. \pm 5.8

^aBALB/c nu/nu and nu/+ mice were infected with LCMV-ARM or LCMV-WE and liver leukocytes were isolated at day 7 p.i. as described in Materials and Methods.

J. Augmentation of liver NK/LGL by other virus infections or poly I:C

The effects of poly I:C or infection by several viruses on liver NK/LGL were compared in different mouse strains (Table 27). C57BL/6J mice treated with poly I:C (Expt. 1) or infected with the non-cytopathic LCMV-ARM (Expt. 2) had comparable increases (2-3-fold compared to controls) in total LGL and in lysis of YAC-1 cells. In vivo treatment with anti-asialo GM₁ serum abrogated NK cell activity and LGL accumulation in both cases (Expt. 1 and 3). However, total numbers of liver leukocytes in the treated animals were only minimally changed from control, whether or not the mice received anti-asialo GM₁ injections.

NK cell-deficient C57BL/6J mice homozygous for the beige mutation (bg/bg) were compared with NK cell-sufficient bg/+ littermates at day 3 p.i. with LCMV-ARM for liver NK/LGL lytic activity and cell number (Expt. 4). C57BL/6J bg/bg mice had markedly reduced NK cell activity associated with liver leukocytes, despite having higher percentages and higher total numbers of LGL than bg/+ mice. However, the majority (76%) of the liver LGL present in the bg/bg mice bore the atypical morphology characteristic of LGL from beige mutant mice (Itoh et al., 1982), i.e., one to three abnormally large cytoplasmic granules rather than the numerous small granules of normal LGL (see Figure 4 for normal morphology). The NK cell activity and accumulation of NK/LGL in the livers of heterozygous bg/+ mice

Table 27

Comparison of Augmentation of Liver NK/LGL by Poly I:C, LCMV-ARM, MCMV, and MHV^a

Expt. Strain	Treatment	% YAC-1 Lysis	Total		Total LGL per Liver (X10 ⁻⁵)
			Leukocytes per Liver (X10 ⁻⁶)	% LGL	
1 C57BL/6J	Control	9.3 ± 2.3	3.6 ± 0.4	9.0 ± 1.4	3.2 ± 0.2
	Poly I:C	31 ± 7.1	3.0 ± 0.8	17 ± 0	5.0 ± 1.3
	Poly I:C + anti-asialo GM1	1.0 ± 0.4	2.5 ± 0.6	1.5 ± 0.7	0.4 ± 0.3
2 C57BL/6J	Control	7.5 ± 3.1	7.9 ± 0.1	4.0 ± 1	4.0 ± 1.4
	LCMV-ARM	16 ± 0.6	12 ± 1.7	4.3 ± 0.6	5.2 ± 0.7
3 C57BL/6J	LCMV-ARM	16 ± 4.6	9.2 ± 2.6	9.0 ± 2.6	7.9 ± 0.8
	LCMV-ARM + anti-asialo GM1	1.8 ± 2	9.9 ± 7.3	2.2 ± 1.3	2.2 ± 1.3
4 C57BL/6J bg/+ bg/bg	LCMV-ARM	15 ± 4	8.9 ± 3.5	8.3 ± 1.2	7.2 ± 1.7
	LCMV-ARM + anti-asialo GM1	2.3 ± 3	13 ± 2.3	11 ± 2.6	14 ± 3.5
5 C57BL/6J	Control	14 ± 0.7	6.7 ± 0.6	4.5 ± 0.7	3.0 ± 0.3
	MCMV	60 ± 4.9	14 ± 0.2	15 ± 1.4	21 ± 2.3
6 C57BL/6J	Control	3.5 ± 2.8	5.1 ± 0.9	3.0 ± 0	1.5 ± 0.3
	MHV	73 ± 4.2	26 ± 9.3	11 ± 2.8	33 ± 5

Table 27 (cont'd)

7	C57BL/6J	Control	10	± 2.2	2.6 ± 0.8	4.5 ± 1	1.2 ± 0.3
		MHV	48	± 3.6	9.5 ± 2.8	12 ± 3	12 ± 5.6
		MHV + anti-asialo GM1	4.2	± 4.9	4.3 ± 0.8	2.3 ± 0.6	2.2 ± 0.7
8	C57BL/6J	Control	0.6	± 0.8	13 ± 2.9	6.5 ± 1.7	8.4 ± 3.2
	bg/bg	MHV	9.3	± 3.5	34 ± 5.1	13 ± 1.5	45 ± 11
9	BALB/cJ	Control	14	± 3.8	14 ± 3.8	3.0 ± 1	4.0 ± 1.4
		LCMV-ARM	39	± 13	23 ± 9.5	5.7 ± 1.5	12 ± 3.3
10	BALB/C	Control	3.0	± 2.1	5.6 ± 1.1	6.0 ± 1.4	3.5 ± 1.5
	nu/nu	Poly I:C	38	± 4.5	5.2 ± 1.1	17 ± 2.5	9.9 ± 3
	nu/nu	LCMV-ARM	44	± 5	6.5 ± 1.1	16 ± 3.1	11 ± 3.9
11	BALB/C	Control	36	± 2	7.5 ± 0.7	16 ± 3	12 ± 2.7
	nu/+	Poly I:C	55	± 5.1	5.0 ± 0.7	27 ± 3.6	14 ± 3.8
	nu/nu	Poly I:C					

^aLiver leukocytes were prepared at day 3 p.i., or 2 days after poly I:C treatment.

(Expt. 4) were similar to those seen in C57BL/6J (+/+) mice (Expt. 2 and 3).

These results showing only modest increases in total leukocyte and LGL number during LCMV infection contrast with the findings at day 3 p.i. in C57BL/6J mice infected with the cytopathic hepatotropic viruses, MCMV (Expt. 5) or MHV (Expt. 6). These virus infections increased the total leukocyte number 2-5-fold, LGL percentages 3-4-fold, and the total LGL number from 7-22-fold. NK cell activity (YAC-1 lysis) was also markedly increased in the MCMV- and MHV-infected mice. As also shown above for poly I:C and LCMV-ARM, anti-asialo GM₁ treatment abolished the NK/LGL increases induced by MHV (Expt. 7). MHV infection of C57BL/6J bg/bg mice also resulted in a significant ($p < .01$) increase in NK/LGL in the liver, but these cells were relatively inefficient in lysing YAC-1 targets (Expt. 8).

NK/LGL responses in normal BALB/cJ and in T cell-deficient nude (nu/nu) BALB/c mice were examined. Following LCMV-ARM infection, BALB/cJ mice (Expt. 9) were found to produce NK/LGL responses comparable to those seen in either C57BL/6J or C3H/St mice. In BALB/c nu/nu mice, poly I:C treatment and LCMV-ARM infection induced similar increases (Expt. 10). When nu/nu and nu/+ mice were compared within the same experiment, poly I:C treatment induced higher YAC-1 lysis and % LGL in nu/nu than in nu/+ mice (Expt. 11). This is in agreement with previous observations of higher levels of endogenous (Stitz et al., 1985)

and activated (Leung and Ada, 1981; Taylor et al., 1985) NK cell activity in nu/nu than in nu/+ mice .

Thus, these experiments (Table 27) demonstrate that NK cell activity and LGL number in the liver are increased early following virus infection or treatment with the IFN-inducer poly I:C. These NK/LGL increases occur in several strains of mice, including strains with genetic defects in NK cell (bg/bg) and T cell (nu/nu) activity.

K. Multiple activation of liver NK cell activity

The ability of sequential treatments with IFN-beta and the IFN-inducer poly I:C to maximize NK/LGL accumulation and lytic activity in the liver was examined. A single injection of IFN-beta (10^5 U) augmented liver NK cell activity and LGL percentage within 24 hr (Table 28). Two treatments (at a four day interval) with IFN or IFN-inducer resulted in maximal LGL accumulation and NK cell activity; a third treatment provided no further increase. These data suggest that NK/LGL may become refractory to repeated stimulation by IFN as has been shown following multiple stimulations of NK cell activity with other biologically active substances (Saito et al., 1985; Talmadge et al., 1985).

L. Kinetics of NK/LGL accumulation during MHV infection

C57BL/6J mice were infected for 1 to 3 days with MHV before leukocytes were isolated from their livers. Table 29 indicates

Table 28

Multiple Activation of Liver NK Cell

Activity by Interferon and Interferon-Inducer

Treatment ^a	% YAC-1	Total Leukocytes	Total LGL	
	Lysis ^b	per Liver ($\times 10^{-6}$)	% LGL	per Liver ($\times 10^{-5}$)
None	2.0	7.5	5.0	3.6
IFN (D-1)	7.5	8.8	12.	11.
Poly I:C (D-5) + IFN (D-1)	16.	12.	14.	17.
Poly I:C (D-9) + IFN (D-5, D-1)	10.	13.	12.	16.

^aBALB/c nu/+ mice (n=2 per group) were untreated or injected i.p. with 100 ug poly I:C and/or injected i.v. with 1×10^5 U IFN-beta on the days indicated before harvest. Liver leukocytes were prepared from all mice on the same day and pooled within groups.

^bYAC-1 assays were run for 4.5 hr at E:T = 33:1.

Table 29
Kinetics of NK/LGL Accumulation During MHV Infection

Day Postinfection ^a	% YAC-1 Lysis ^b	Total Leukocytes per Liver ($\times 10^{-6}$)	% LGL	Total LGL per Liver ($\times 10^{-5}$)
0 (control)	12.±6.5	3.9 ± 1.3	4.7±0.6	1.9 ± 0.8
1	25.±3.5	2.0 ± 0.1	6.0±1.0	1.2 ± 0.1
2	27.±2.0	3.0 ± 0.4	8.7±3.1	2.7 ± 1.2
3	36.±6.7	6.0 ± 1.4	12. ±4.0	6.7 ± 1.5

^aC57BL/6J mice (n=3 per group) were uninfected (Day 0 control) or infected i.p. with MHV. Liver leukocytes were prepared from all mice on the same day.

^bYAC-1 assays were run for 5 hr at E:T = 7:1.

that as early as day 1 p.i. an increase in NK cell activity (YAC-1 lysis) was evident, despite no increase in LGL percentage. This suggests that the increased lytic activity was due to activation of NK cells by virus-induced IFN and not by increased proportions of NK cells in the leukocyte populations. LGL percentage began to rise noticeably by day 2 p.i., with NK cell activity, LGL percentage, and total LGL number reaching high levels by day 3 p.i.

M. Virus vs NK/LGL: dose-response relationships

C57BL/6J mice were infected for 3 days with varying doses (PFU) of MCMV. The data in Table 30 show that the NK/LGL response to MCMV was, in at least some parameters, biphasic. Increasing the MCMV dose from 2×10^4 to 10×10^4 PFU led to increasing numbers of total leukocytes and total LGL. However, even the lowest virus dose tested (2×10^4 PFU) produced near-maximal NK cell activity and LGL percentages. At high doses of 50×10^4 to 100×10^4 PFU, all measured response parameters began to decline. As MCMV is known to be capable of inducing immunosuppression in mice (Allan et al., 1982; Loh and Hudson, 1982), immunosuppression seems a likely explanation for the reduced NK/LGL responses at the higher doses.

Table 30
Effect of MCMV Dose on Liver NK/LGL Accumulation

Virus Dose (PFUx10 ⁴)	% YAC-1 Lysis ^b	Total		Total LGL Per Liver (x10 ⁻⁵)
		Leukocytes Liver (x10 ⁻⁶)	% LGL	
None	3.1 ± 0.9	6.7 ± 0.6	4.5 ± 0.7	3.0 ± 0.3
2	30. ± 4.2	7.4 ± 0.5	20. ± 2.1	14. ± 2.5
5	31. ± 5.7	9.8 ± 3.6	21. ± 2.1	20. ± 5.4
10	29. ± 2.8	14. ± 0.2	13. ± 1.4	21. ± 2.3
50	26. ± 9.2	10. ± 2.3	9.0 ± 1.4	9.1 ± 3.5
100	20. ± 6.4	12. ± 0.6	7.5 ± 0.7	9.0 ± 1.2

^aC57BL/6J mice (n=2 per group) were uninfected i.p. with varying doses of MCMV. Liver leukocytes were prepared at day 3 p.i.

^bYAC-1 assays were run for 4 hr at E:T = 3:1.

CHAPTER VI

BLASTOGENESIS AND PROLIFERATION OF LGL IN THE LIVER

Data in the previous chapter demonstrate that high numbers of LGL accumulate in the liver during the course of viral infection. It has been recently shown (McIntyre et al., 1987) that the accumulation of total numbers of NK/LGL in the virus-infected peritoneal cavity was severely reduced if mice were irradiated prior to infection. This suggested that cell proliferation was necessary for the accumulation of normal numbers of LGL. The experiments presented below were conducted to examine the potential contribution of blastogenesis and proliferation of LGL to cell-mediated immune responses to viral infection of the liver.

A. Effect of shielding the bone marrow against lethal irradiation on NK/LGL response to MHV infection

NK cell precursors are found predominantly in the bone marrow (see Chapter I). The effects of protecting the bone marrow environment from lethal irradiation on subsequent NK/LGL responses to viral infection were examined. Table 31 shows that shielding of the bone marrow resulted in NK/LGL responses that were essentially no different from animals receiving total body irradiation. The LGL percentage was actually significantly higher ($p < .01$) in leukocytes from total body-irradiated mice

Table 31
 Effect of Shielding the Bone Marrow Against
 Lethal Irradiation on the NK/LGL Response to MHV Infection^a

<u>Irradiation</u> <u>Treatment</u>	<u>% YAC-1</u> <u>Lysis</u> ^b	<u>Total Leukocytes</u> <u>(x 10⁶)</u>	<u>% of</u> <u>Leukocytes</u> <u>with LGL</u> <u>Morphology</u>	<u>Total LGL</u> <u>(x 10⁵)</u>
Total body	17.±4.8	1.8 ± 0.4	11. ± 1.4	2.0 ± 0.6
Bone marrow- shielded	18.±3.8	2.4 ± 0.8	8.0± 0.9	2.0 ± 0.6

^aMice (n=6 per group) received 900 R electron irradiation with or without shielding of fore- and hindlimbs as described in Materials and Methods. Mice were then immediately infected with MHV, and liver leukocytes were obtained 3 days p.i.

^bYAC-1 assays were 4 hr at E:T = 10:1.

than in leukocytes from bone marrow-shielded mice. This was apparently due to higher percentages of polymorphonuclear leukocytes (which "diluted out" the LGL) in the bone marrow-shielded populations compared to total body-irradiated populations (23% vs 9.7%, respectively; $p < .02$).

B. Role of the spleen in NK/LGL response to MHV infection

Since the spleen is a site of mature NK cell blastogenesis during viral infection (Biron and Welsh, 1982), it was hypothesized that the spleen might be providing a significant number of the LGL accumulating in the liver. The ability of the spleen alone to provide NK/LGL for the early immune response was examined. Mice were anesthetized and their spleens were isolated outside the abdominal wall under lead shielding. Electron irradiation (900 R) was then administered to these mice and to similarly operated mice without lead-shielded spleens. The shielded spleens contained 7-20-fold greater numbers of viable leukocytes than did the irradiated spleens at day 3 postirradiation (data not shown). Table 32 indicates that, in those animals whose spleens were spared from the irradiation, NK/LGL responses in the liver to MHV infection were not significantly greater than in those animals whose spleens were irradiated. Preserving the proliferative capacity of splenic NK/LGL did not alter the deleterious effects of lethal irradiation of other lymphoid compartments. However, other

Table 32
 Effect of Shielding the Spleen Against
 Lethal Irradiation on the NK/IgL Response to MHV Infection^a

Irradiation Treatment	% YAC-1 Lysis ^b	Total Leukocytes (x10 ⁶)	% of	
			Leukocytes with IgL Morphology	Total IGL (x10 ⁴)
Total body	19. ± 3.0	2.8 ± 0.6	20. ± 1.0	56. ± 14.
Spleen shielded	26. ± 10.	3.5 ± 0.9	17. ± 1.5	60. ± 10.

^aMice received 1000 R electron irradiation with or without shielding of their spleens as described in Materials and Methods. Mice were then immediately infected with MHV, and liver leukocytes were obtained 3 days p.i.

^bYAC-1 assays were 5 hr; E:T = 3:1.

effects of irradiation such as altered lymphocyte trafficking patterns or the generation of factors inhibiting proliferation of viable cells could have accounted for these results as well as the results obtained in the bone marrow shielding experiments above. Because of this uncertainty, a splenectomy model was used to examine the contribution of NK/LGL made by the spleen. Mice were splenectomized or sham-operated and then infected with MHV. At day 3 p.i., the absence of a spleen did not appreciably alter the NK/LGL response in the liver (Table 33). Thus, by two different methods, a significant role of the spleen in providing NK/LGL to this non-lymphoid compartment could not be demonstrated.

C. LGL with blast cell morphology in freshly isolated liver leukocytes

Careful scrutiny of stained cytocentrifuge smears made from virus-activated liver leukocyte populations revealed the presence of considerably enlarged LGL with intensely basophilic cytoplasm and often conspicuous nucleoli (Figure 4). This morphology was consistent with that usually attributed to blast lymphocytes (Hayry and von Willebrand, 1981). Occasionally, mitotic figures were seen which contained prominent azurophilic cytoplasmic granules (Figure 4). Since these leukocytes were freshly isolated from the liver and not cultured in vitro, their presence suggested that LGL in the liver were undergoing blastogenesis and

Table 33
NK/LGL Response to MHV Infection in Splenectomized Mice^a

<u>Treatment</u>	<u>% YAC-1 Lysis</u> ^b	<u>% of Leuko- cytes with LGL Morphology</u>	<u>Total LGL (x 10⁵)</u>
Sham	27. ± 8.1	7.3 ± 1.3	7.6 ± 1.7
Splenectomy	31. ± 11.	7.0 ± 2.2	9.1 ± 2.1

^aC57BL/6J mice were sham-operated or splenectomized as described in Materials and Methods, and then immediately infected with MHV. Liver leukocytes were harvested at day 3 p.i.

^bYAC-1 assays were run for 4 hr at liver E:T = 17:1.



Figure 4

Figure 4. LGL and blast LGL isolated from the livers of virus-infected mice. A. Endogenous LGL. B. Blast LGL and endogenous LGL. C. Mitotic figure with cytoplasmic granules (mitotic LGL). Wright's and Giemsa, X 400.

proliferation.

D. Size fractionation of blast LGL

Viral infection or exposure to IFN in vivo elicits the blastogenesis of spleen NK cells with a concomitant increase in cell size (Biron and Welsh, 1982; Biron et al., 1984). Upon size separation by centrifugal elutriation, ^3H -thymidine-incorporating blast spleen cells are contained almost exclusively within the large-cell fractions 4, 5, and 6 (Biron and Welsh, 1982). Centrifugal elutriation was here employed to separate poly I:C-stimulated liver leukocytes into populations of increasing size, and the number of leukocytes recovered in each fraction was determined. Cytocentrifuge smears of each fraction were analyzed by light microscopy for the proportion of LGL exhibiting non-blast or blast cell morphology (as defined in Chapter II). The total number of non-blast LGL and blast LGL in each fraction was then calculated. Figure 5 shows that the majority (81%) of the LGL exhibiting blast cell morphology were recovered in the large cell fractions (fractions 4-6). The small cell fractions (fractions 1-3) contained 61% of all non-blast LGL recovered and had few LGL that were blast LGL. Thus, these data show that morphological criteria could be used to classify LGL into blast and non-blast populations and that poly I:C elicited LGL with blast size and morphology in the liver.

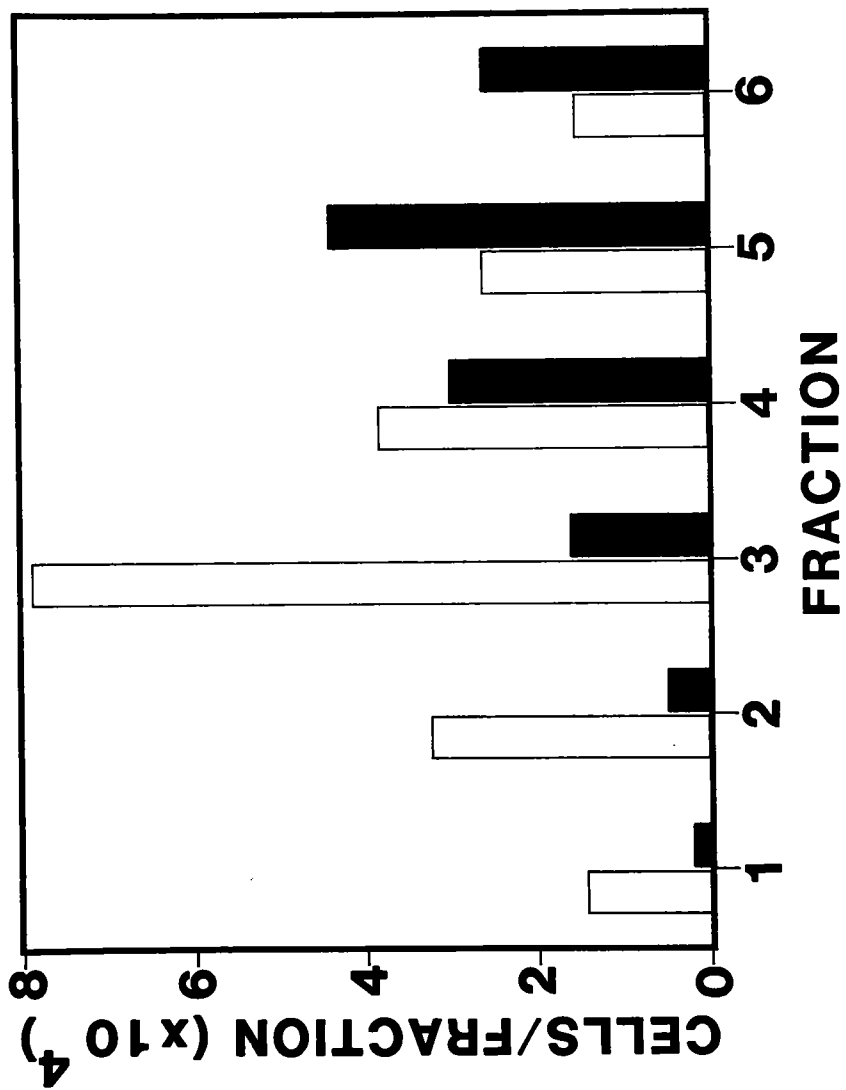


Figure 5

Figure 5. Size separation of blast LGL by centrifugal elutriation. Leukocytes from the livers of poly I:C-treated mice were separated into fractions of increasing cell size (fractions 1 through 6, respectively) by centrifugal elutriation as described in Materials and Methods. The total number of leukocytes in each fraction was determined. The percentage of leukocytes in each fraction exhibiting non-blast LGL morphology and blast cell/mitotic figure LGL morphology was assessed by differential counts of Wright'-Giemsa stained cytocentrifuge smears. The total number of non-blast LGL (OPEN BARS) and blast LGL (SOLID BARS) recovered in each fraction was then calculated.

E. Association of blast LGL with augmented NK cell activity

As shown in Table 34, increases in NK cell activity (YAC-1 cell lysis) and in the number of LGL in livers following MHV infection were associated with increases in the proportion of those LGL exhibiting a blast cell morphology. LGL in the liver at this time (day 3 p.i.) have been previously well-characterized as NK cells on the basis of cell surface phenotype and lytic profile (see Chapter V). Treatment of mice with anti-asialo GM₁ serum, which selectively depletes NK cell activity in vivo (Habu et al., 1981), abrogated the majority of the virus-induced increases in blast LGL as well as non-blast LGL (Table 34). These data suggest that the blast LGL were NK cells.

F. Thymidine-incorporating killer cells in the liver

The presence of morphologically-defined blast LGL in leukocyte populations freshly isolated from the liver during viral infection suggested that cytotoxic lymphocytes were proliferating at this site. To confirm that the specific cells mediating the killing were indeed blast cells, non-adherent leukocytes from livers of control mice and MHV-infected mice at day 3 p.i. were labeled with ³H-thymidine and tested in single cell cytotoxicity assays with autoradiography. The data in Table 35 show that MHV induced significant increases in NK cell-mediated lysis and in the percentage of LGL with blast cell morphology. Leukocytes from infected mice which had bound a target were more lytically

Table 34

Blast LGL in Liver Leukocyte Populations at Day 3 Postinfection^a

Liver	% of YAC-1		% of Leuko- cytes with		Total LGL (x10 ⁵)	% of LGL with Blast		Total Blast LGL (x10 ⁴)
	Lysis ^b		LGL Morphology			Morphology ^c		
Control	2.9 ± 0.7		4.5 ± 1.0		1.2 ± 0.3	5.5 ± 2.5		0.7 ± 0.4
MHV	28. ± 1.7		12. ± 3.0		12. ± 3.0	46. ± 1.5		53. ± 26.
MHV + anti-AGM1	0.2 ± 2.1		2.3 ± 0.6		2.2 ± 0.6	28. ± 7.4		12. ± 3.3

^aLiver leukocytes were harvested at day 3 p.i. with MHV. Some mice were treated with 0.2 ml of a 1:10 dilution of anti-asialo GM1 serum at the time of infection.

^bYAC-1 assays were 5 hr; liver E:T = 3:1.

^c% of the LGL with mitotic or blast morphology on cytocentrifuge preparations, as described in Materials and Methods.

Table 35

Thymidine-Incorporating NK Cells in the Liver During Viral Infection

	% of YAC-1 <u>Lysis^a</u>	% of Leuko- cytes with <u>LGL Morphology</u>	% of LGL with Blast <u>Morphology</u>	³ H-Thymidine <u>Incorp. (CPM)^b</u>	% Cytotoxicity <u>in Conjugates^c</u>	% Killing Mediated by ³ H-Labeled <u>Effector Cells^c</u>
Control	16.	12.	20.	2847	13.	14.
MHV	50.	38.	56.	5654	30.	25.

^aFour hr assay; liver E:T = 3:1.

^bCPM/10⁵ nonadherent leukocytes following 1 hr pulse with ³H-thymidine.

^cFrom single cell assays as described in Materials and Methods.

active (% killing in conjugates) compared to cells from control mice. Most importantly, a significantly ($p < .001$) greater percentage of the lytic activity was mediated by ^3H -thymidine-incorporating effector cells (% killing mediated by blast cells) from infected mice than from uninfected mice. Thus, compared to uninfected control leukocytes, day 3 p.i. leukocyte populations from the liver incorporated more ^3H -thymidine, had higher proportions of LGL demonstrating blast cell morphology, and had significantly increased numbers of ^3H -thymidine-incorporating leukocytes that bound to and lysed NK cell-sensitive targets. These data show that leukocytes freshly isolated from this non-lymphoid compartment at day 3 p.i. contain ^3H -thymidine-incorporating, functionally active, blast NK cells.

G. Blast NK/LGL are induced by several virus infections

The presence of blast NK/LGL in the leukocytes isolated from liver was examined in several mouse strains infected with different viruses. Viral infection resulted in significant increases in both the percentages and total numbers of blast NK/LGL at day 3 p.i. (Table 36). The liver-derived blast (and non-blast) NK/LGL at day 3 p.i. were significantly depleted ($p < .05$) by in vivo treatment with anti-asialo GM_1 serum. Two immunodeficient strains of mice were also examined for the induction of blast NK/LGL in the liver. T cell-deficient nude (nu/nu) mice generated large numbers of blast NK/LGL in response to LCMV

Table 36

Blast LGL in the Liver 3 Days After Viral Infection^a

<u>Mouse Strain</u>	<u>Treatment</u>	<u>% of LGL with Blast Morphology</u>	<u>Total Blast LGL (x10⁴)</u>
C57BL/6J	Control	8.5 ± 3.5	2.5 ± 0.8
	MCMV	70. ± 2.1	149. ± 21.
C57BL/6J	LCMV-ARM	31. ± 4.0	25. ± 3.8
	LCMV-ARM + anti-AGM1	31. ± 6.4	7.2 ± 5.9
C3H/St	LCMV-WE	64. ± 2.3	28. ± 2.1
	LCMV-WE + anti-AGM1	53. ± 2.0	5.3 ± 1.3
C57BL/6J bg/bg	Control	4.3 ± 2.1	3.6 ± 2.0
	MHV	42. ± 6.2	188. ± 53.
BALB/cJ	Control	10. ± 2.1	4.3 ± 2.2
	LCMV-ARM	44. ± 3.5	53. ± 13.
BALB/c nu/nu	Control	5.0 ± 2.8	1.9 ± 1.2
	LCMV-ARM	50. ± 8.7	54. ± 30.
nu/nu	Poly I:C	59. ± 3.5	52. ± 19.

^aCyocentrifuge preparations of leukocytes from the livers of mice (n=3-5 per group) at day 3 post-infection were examined for the proportion of LGL with blast cell morphology, as described in Materials and Methods.

infection as well as to treatment with the interferon-inducer poly I:C (Table 36), suggesting a major role for interferon in the blastogenesis of NK/LGL in the liver. Beige (bg/bg) mutant mice, which are deficient in NK cell activity (Roder and Duwe, 1979), nevertheless responded to MHV infection with vigorous LGL blastogenesis (Table 36). The majority ($81. \pm 2.1 \%$) of the bg/bg blast NK/LGL at day 3 p.i., however, had abnormal granule morphology, i.e., one to three large granules, typical of beige NK/LGL (Itoh et al., 1982).

H. Kinetics of LGL blastogenesis

The kinetics of LGL blastogenesis in the peritoneal cavity has been examined during MHV infection (McIntyre et al, 1987), where both the percent of the LGL that were blasts and the total numbers of blast LGL in the peritoneal cavity peaked at day 3 p.i. and then declined rapidly. By day 7 p.i. with MHV, LGL blastogenesis and total LGL number had returned to control values (McIntyre et al, 1987).

The kinetics of LGL blastogenesis in the liver was examined following infection of C3H/St mice with the hepatotropic WE strain of LCMV (LCMV-WE) or the non-hepatotropic Armstrong strain of LCMV (LCMV-ARM). Increases in blast LGL proportion were evident as early as day 1 p.i. with both viruses (Figure 6), but, in contrast to MHV-induced blastogenesis (McIntyre et al., 1987), LCMV-induced blastogenesis remained elevated through day 7 p.i.

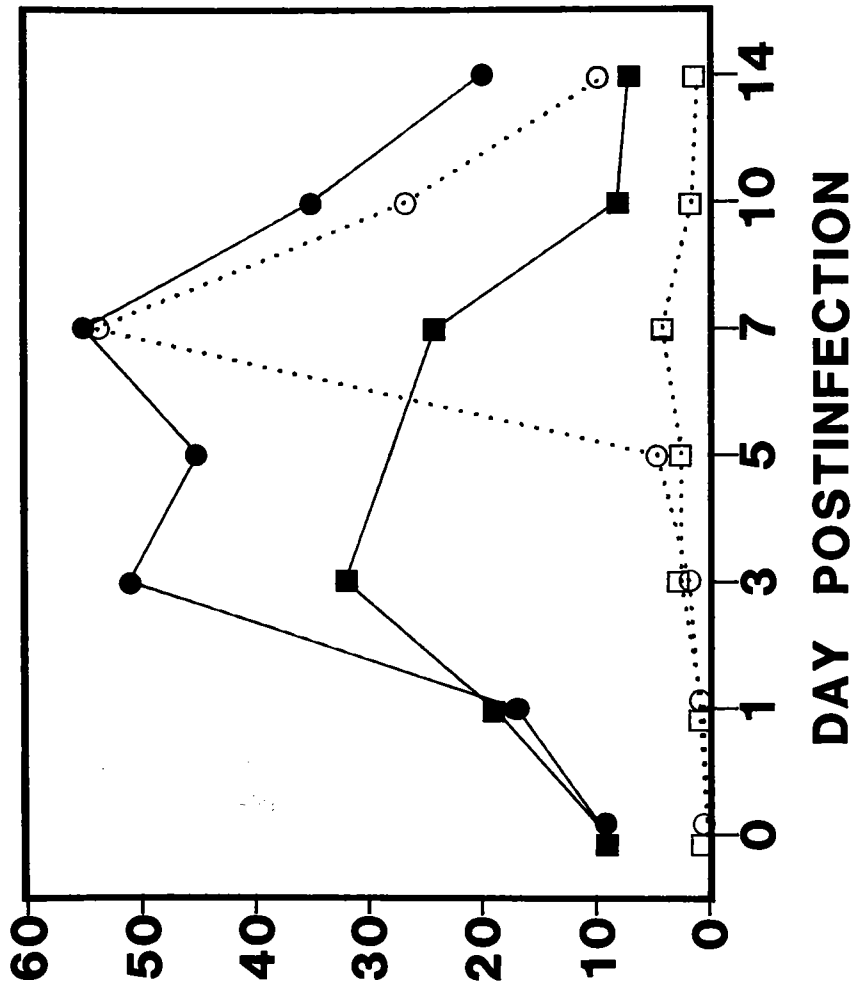


Figure 6

Figure 6. Kinetics of LGL blastogenesis during LCMV infection. Mice (n=2-4 per group) were infected with LCMV-ARM (□ , ■) or LCMV-WE (○ , ●) and liver leukocytes were prepared at the indicated times postinfection. Percent of the LGL exhibiting blast morphology (———). Number of blast LGL ($\times 10^5$) per liver (-----).

before declining. This was a difference between LCMV and MHV, not of liver and peritoneal cavity, as the peritoneal blast LGL response to LCMV-ARM resembles that of the liver (Biron et al., 1986; Dr. R. Natuk, personnel communication). Compared to infection with LCMV-ARM, infection with LCMV-WE resulted in significantly ($p < .02$) higher percentages of blast LGL in liver leukocytes by day 3 p.i., and this difference persisted through day 14 p.i. The total number of blast LGL in livers infected with either LCMV-ARM or LCMV-WE peaked at day 7 p.i., but these levels were 13-fold higher following hepatotropic LCMV-WE infection.

I. Resistance to anti-asialo GM1 serum of day 7 p.i. blast LGL

In the spleen during LCMV infection, NK cells undergo peak blastogenesis at day 3 p.i. (Biron and Welsh, 1982) and CTL undergo peak blastogenesis at day 7 p.i. (Biron et al., 1986). The prolonged LGL blastogenesis in the liver during LCMV infection (Figure 6) suggested that a portion of the blast LGL present, especially later in infection, could be of the CTL phenotype. Since blast NK/LGL at day 3 p.i. were sensitive to depletion by in vivo treatment with anti-asialo GM1 serum (Tables 34 and 36), the sensitivity of blast LGL at day 7 post-LCMV infection to depletion by anti-asialo GM1 was tested. In contrast to the findings at day 3 p.i., two treatments with anti-asialo GM₁ serum in vivo did not reduce day 7 p.i. LGL

blastogenesis in C57BL/6J mice infected with LCMV-ARM or in C3H/St mice infected with LCMV-WE (Table 37). These treatments significantly depleted NK cell activity (YAC-1 lysis) but not LCMV-specific CTL activity.

J. Lyt-2 phenotype of blast LGL

The blast LGL populations from the livers of mice at days 3 and 7 p.i. with LCMV were examined by FACS for expression of the CTL-specific marker Lyt-2. At day 3 p.i., the majority of the LGL (77%), blast LGL (81%) and NK cell activity was found in Lyt-2⁻ FACS-sorted populations from the liver (Table 38). By contrast, most LGL (58%) and blast LGL (82%) in the liver at day 7 p.i. were Lyt-2⁺. NK cell activity at day 7 p.i. was still mediated by Lyt-2⁻ cells. Lyt-2⁺ cells, which were devoid of CTL activity at day 3 p.i., expressed high levels of virus-specific (CTL) lysis at day 7 p.i. (Table 38). These data indicate that LGL blastogenesis shifted from NK cell-rich, Lyt-2⁻ leukocytes at day 3 p.i. to CTL-rich, Lyt-2⁺ leukocytes at day 7 p.i. Even though Lyt-2⁻ LGL, presumed to be NK cells, were still present at day 7 p.i., few of them were dividing.

Table 37

Resistance of Blast LGL at Day 7 Post-LCMV Infection to Anti-Asialo GM₁^a

Mouse Strain	Treatment	% of LGL with		Total Blast		% Virus-	
		Blast Morphology	LGL (x 10 ⁴)	% YAC-1 Lysis ^b	Specific Lysis ^c		
C57BL/6J	LCMV-ARM	44. ± 1.2	111. ± 9.8	7.4 ± 0.5	53. ± 3.2		
	LCMV-ARM + anti-AGM1	62. ± 2.0	231. ± 51.	1.9 ± 0.3	66. ± 4.9		
C3H/St	LCMV-WE	56. ± 2.3	484. ± 100.	6.2 ± 0.8	50. ± 1.0		
	LCMV-WE + anti-AGM1	66. ± 0.0	467. ± 62.	2.2 ± 0.5	57. ± 1.2		

^aLeukocytes were isolated from livers of LCMV-infected mice at day 7 p.i. Mice were treated with

0.2 ml of anti-asialo GM₁ (1:10 dil) i.v. on days 0 and 4 p.i. where indicated.

^bYAC-1 assays were run for 4 hr at E:T = 3:1.

^c% Virus-specific lysis is the difference in lysis between LCMV-infected and uninfected histocompatible target cells determined in 16 hr assays at E:T = 10:1.

Table 38

Lyt-2 Phenotype of Blast IGL at Days 3 and 7 Postinfection with LCMV-ARM

Cell Population ^a	% of Leuko- cytes with		% of IGL With Blast Morphology ^c	% Virus-Specific		% YAC-1 Lysis ^e
	LGL Morphology ^b			Lysis ^d		
Day 3 Lyt-2 ⁺ (11.)	34. (23.)		34. (19.)	< 1		11.
Lyt-2 ⁻ (89.)	13. (77.)		48. (81.)	< 1		20.
Day 7 Lyt-2 ⁺ (27.)	42. (58.)		44. (86.)	60.		6.2
Lyt-2 ⁻ (73.)	13. (42.)		10. (14.)	16.		32.

^aLeukocytes were obtained from the livers of C3H/St mice at days 3 and 7 p.i. with LCMV-ARM. Liver leukocytes were stained with anti-Lyt-2 antibody and sorted by FACS as described in Materials and Methods. Values in parentheses show the percent of leukocytes exhibiting the indicated Lyt-2 phenotype.

^b% of the FACS-sorted cells with IGL morphology. Values in parentheses show the percent of all IGL exhibiting the indicated Lyt-2 phenotype.

^c% of the FACS-sorted IGL with blast cell/mitotic figure morphology. Values in parentheses show the percent of all blast IGL exhibiting the indicated Lyt-2 phenotype.

^d% Virus-specific lysis was the difference in lysis between LCMV-infected and uninfected L-929 cells in 14 hr assays at E:T = 10:1.

^eYAC-1 assays were run for 4 hr at E:T = 10:1.

CHAPTER VII

DISCUSSION

The data in this thesis demonstrate morphologically, phenotypically, and functionally the accumulation of cytotoxic effector lymphocytes in the liver during viral hepatitis. Adoptive transfer experiments show that spleen cell populations containing virus-specific CTL most likely mediate their antiviral effects in vivo by a mechanism of direct cytolysis. Thus, the correlation of in vivo antiviral cytotoxic function with the in situ accumulation of cells displaying a cytotoxic morphology (LGL) strengthens the proposals that reductions in infectious virus titers and that tissue pathology in certain viral diseases are cytotoxic cell-mediated immune phenomena.

A. Regulation of LCMV infection by cytotoxic lymphocytes

A growing body of evidence (Welsh and Kiessling, 1980; Bukowski et al., 1983b; Bukowski et al., 1984; Bukowski et al., 1985) has indicated, either directly or indirectly, that NK cells play no apparent role in resistance to LCMV infection. Similar conclusions have been drawn from studies with Sindbis virus (Hirsch, 1981). The findings in the LCMV system were confirmed and extended here to show that acute infection with the strongly hepatotropic LCMV-WE strain resulted in equivalent levels of

viral replication in the presence or absence of NK cells (Table 1). This suggested that the very limited replication of LCMV-ARM in certain organs (such as the liver) during acute infection was not responsible for its resistance to NK cell-mediated antiviral effects. A more likely explanation for this resistance was that the IFN generated during the early course of LCMV infection (Welsh, 1978a) protected LCMV-infected target cells in vivo from activated NK cell-mediated lysis (Bukowski and Welsh, 1985b). Additionally, NK cell depletion by anti-asialo GM1 treatment of mice persistently infected with LCMV-ARM (which grows to high titer in many organs, including the liver) did not affect virus synthesis (Table 2), confirming earlier studies by Bukowski et al (1983a). However, since persistently infected mice make an antibody response to LCMV (Oldstone and Dixon, 1967), the presence of anti-LCMV antibody in these mice may have greatly overshadowed, in the short term of the observation period, a minor contribution of NK cells to the control of the persistent infection.

Although a direct anti-LCMV effect of NK cells could not be shown, the possibility of NK cells exerting an antiviral function via ADCC in LCMV infection was examined. NK cells express on their surface receptors for the Fc portion of immunoglobulin (Herberman et al., 1977), and consequently are capable of mediating ADCC (Ojo and Wigzell, 1978; Bradley and Bonavida, 1982). ADCC has been suggested as a mechanism of antiviral

resistance in passively immunized, Sindbis virus-infected mice (Hirsch et al., 1979). A role for NK cell-mediated ADCC was tested by passively immunizing naive mice with anti-LCMV antibody then infecting them with LCMV in the presence or absence (anti-asialo GM1-treatment) of NK cells. By this technique, an antibody-dependent, NK cell-mediated antiviral activity could not be demonstrated (Table 4). Use of the less specific immunosuppressant cyclophosphamide (CY) in a similar passive (antibody) protection protocol again did not reveal an antibody-cellular immune system interaction (Table 5). Interestingly, mice treated with CY alone had lower titers of LCMV than infected control mice (Table 6). This suggested that the CY treatment may have depleted a cell population that would otherwise have supported viral replication.

A portion of the antiviral effect of immune serum transfer did, however, appear to involve components of the C system, as cobra venom factor (CVF)-treated, passively immunized mice had higher virus titers than mice receiving anti-LCMV antibody alone (Table 6). The C system has been implicated as playing a significant role in antibody-mediated protection (Hirsch et al., 1978; Schmaljohn et al., 1982) as well as in antibody-mediated tissue damage (Oldstone and Dixon, 1971) during acute viral infection. Complement-mediated, antibody-dependent lysis of virions and/or LCMV-infected cells is likely responsible for the results obtained here.

Mice acutely infected with LCMV receiving virus-immune spleen cells at early stages of infection rapidly clear virus in the spleen and other organs (Zinkernagel and Welsh, 1976). Adoptive immunization of mice infected with LCMV required theta-bearing lymphocytes from LCMV-immune mice syngeneic with recipient mice in the K or D, but not I, regions of the H-2 locus of the major histocompatibility complex (MHC) (Zinkernagel and Welsh, 1976). Since CTL have similar K/D recognition patterns, it was suggested that the adoptive immunization may be mediated by CTL which lyse the virus-infected target cells in vivo (see Chapter I). Byrne and Oldstone (1984) have recently shown that LCMV-specific cloned CTL can also mediate adoptive immunization.

However, upon recognition of an appropriate target, T cells secrete a number of soluble factors including immune (gamma) IFN (Morris et al., 1982), macrophage migration inhibition factor (Newman et al., 1978), and lymphotoxins (Hiserodt et al., 1979). Gamma IFN has been shown to markedly inhibit virus synthesis (Wheelock, 1965), to activate NK cells (Kumar et al., 1979), and to activate macrophages (Schultz and Chirigos, 1979). All these factors could contribute to the resistance to various virus infections. It is possible, therefore, that the antiviral effect seen during adoptive immunization is not due to direct and specific cytotoxicity, but to nonspecific events that follow triggering of the T cell by antigen recognition.

Previous studies had shown that vaccinia virus-immune

splenocytes failed to immunize against LCMV infection, but they were transferred into mice only infected with LCMV and were unlikely to be stimulated in that environment (Zinkernagel and Welsh, 1976). The experiments described here, however, were designed to ensure that the transferred immune T cells were reactive with antigens in the recipient mice. Alloimmune CTL transferred into LCMV-infected recipients expressing the foreign MHC antigens to which the immune cells had been primed exerted no demonstrable antiviral effect (Table 8). However, *in vivo* activity of these cells was not documented.

The experiments utilizing dually infected recipient mice provided more conclusive results. Using a dual infection model with totally unrelated viruses (LCMV and vaccinia virus), significant reductions in the titers of only the virus which sensitized the transferred spleen cells were noted, with no significant effect on the titers of the "bystander" heterologous virus (Table 12).

Pichinde virus (PV) was substituted for vaccinia virus in later studies of adoptive immunization of dually infected mice. As PV is an arenavirus closely related to, but antigenically distinct from, LCMV (Buchmeier et al., 1981), this combination would require a high degree of specificity on the part of the adoptively transferred immune cells to mediate a selective reduction in titer of only one of the two viruses. As shown in Table 16, reduction of titers in mice infected with only one

virus was, as suspected, mediated only by the relevant virus-immune splenocytes. In mice dually infected with LCMV and PV, LCMV titers were reduced upon transfer of LCMV-immune splenocytes, and PV titers were reduced by PV-immune splenocytes; in neither instance was a significant reduction in the titer of the heterologous virus observed (Tables 16 and 17).

This exquisite specificity in adoptive immunization was somewhat surprising. One might expect that virus in the vicinity of other viruses to which the immune response is directed may be affected by non-specific lymphokines. However, only H-2K or H-2D compatibility is required for adoptive immunization in the LCMV system (Zinkernagel and Welsh, 1976), and an LCMV-specific, H-2-restricted CTL clone can successfully transfer adoptive immunity in LCMV-infected histocompatible mice (Byrne and Oldstone, 1984). This indicates that cells with cytotoxic T cell phenotypes and recognition patterns mediate antiviral effects *in vivo*, but still do not discriminate between direct *in vivo* cytotoxic effects and potential non-specific antiviral effects mediated by soluble factors.

A report by Lukacher et al (1984) has demonstrated specific virus reduction in the lungs of dually infected mice following adoptive transfer of anti-influenza virus CTL clones. In mice infected with two influenza A subtypes, a subtype-specific clone reduced virus titers only of the recognized subtype. A subtype cross-reactive clone lowered titers of both subtypes. The in

vivo reactivity of these clones mirrored their in vitro cytolytic reactivities. This work, like the present studies, is consistent with a highly localized effect of the T cells, such as direct cytotoxicity. However, prior success in the influenza virus CTL clone adoptive transfer model has been highly variable, with some clones providing in vivo protection (Lin and Askonas, 1981) while others do not (Taylor and Askonas, 1983), despite showing in vitro cytolytic specificity. Therefore, the inhibition of virus replication seen in the adoptive transfer models using cloned CTL lines may represent specificity phenomena peculiar to the individual clones used. The present data, obtained following adoptive transfer of immune whole spleen cells, may more closely reflect the normal antiviral activity of the specific immune response of the infected host.

Thus, two classes of potent cytotoxic effectors, NK cells and CTL, are generated during LCMV infection. Despite the conclusively demonstrated role of NK cells in providing protection against infection with certain other viruses (Bukowski et al., 1985), no such role for NK cell-mediated antiviral resistance has been shown in LCMV infection. This pertains to NK cell natural cytotoxicity as well as to NK cell-mediated ADCC in both acute and persistent LCMV infections. However, the results demonstrating an exquisite specificity in virus reduction by transferred immune cells strongly support the concept that T

cell-dependent adoptive immunization against arenavirus infections involves direct in vivo cytotoxic effects by the virus-immune splenocytes. A highly restricted and extremely localized, but virus-non-specific, antiviral effect following antigen recognition by the immune splenocytes is another possible, but less likely, explanation for the data.

B. Cell-mediated immunity in viral hepatitis

These data demonstrate for the first time the accumulation of both NK/LGL and CTL/LGL in the liver during a virus infection. The accumulation of these cells correlates with the appearance of NK cell and CTL cytotoxic activities. During the early course of infection, cytopathic viruses induce more extensive NK/LGL responses in the liver than do non-cytopathic viruses. Infection with the hepatotropic LCMV-WE strain results in a significantly greater CTL/LGL response in the liver on day 7 p.i. than does infection with the non-hepatotropic LCMV-ARM strain. Zinkernagel et al (1986) have recently presented similar results confirming the ability of LCMV-WE infection (compared to LCMV-ARM infection) to induce higher levels of CTL activity and total numbers of inflammatory cells in the livers of mice, correlating with more extensive clinical disease.

The NK cell activity in the liver is similar to the NK cell activity reported in the spleen during LCMV infection (Welsh, 1978a). The phenotype of the liver-derived NK cell is asialo

GM_1^+ , Thy 1.2⁺/⁻, and J11d⁻, and the cell is contained within a population of leukocytes bearing the LGL morphology (Table 20). Liver NK/LGL activated in vivo by poly I:C treatment could be enriched in plastic non-adherent cell populations (Table 23). The early appearance (day 1-2 p.i.) of augmented NK cell lytic activity in the liver is also seen in the spleen (Welsh, 1978a) where activity peaks around day 3 p.i. and then declines. Furthermore, the liver leukocytes contain "activated" NK cells at day 3 p.i. as evidenced by their ability to lyse L-929 cells, a target resistant to "endogenous" NK cell-mediated lysis (Figure 2D). The lysis of NK cell-sensitive YAC-1 cells by liver leukocytes continues to remain above endogenous levels on day 7 p.i. with LCMV-ARM (Figure 2E), but lysis of the more resistant L929 cells is declining (Figure 2D). This prolonged elevation of NK cell-mediated lysis against YAC-1 cells has also been observed in peripheral blood (Stitz et al., 1985). The circulating NK cells at day 7 p.i. are lytically active against YAC-1 targets but they have lost their ability to lyse the more resistant L-929 cells (Stitz et al., 1985). Thus, the high NK cell-mediated lysis against YAC-1 cells seen in the liver and blood at day 7 p.i. is more likely due to the high NK cell number rather than to an activated state of the NK cell population. This may reflect the fact that IFN levels, required for NK cell activation, decline after 3 days postinfection (Welsh, 1978a).

The early cellular (NK/LGL) immune responses in the liver

following non-hepatotropic LCMV-ARM and hepatotropic LCMV-WE infections are quite similar, with NK cell activities and LGL numbers being relatively parallel for the two infections through day 5 p.i. (Figure 2). This may indicate that the augmentation of NK cell activity and LGL number in the liver is due to systemic effects of IFN rather than to virus replication in the liver. The IFN-inducer poly I:C stimulates comparable increases in NK cell activity and LGL number (Table 27). In contrast, MHV and MCMV, which cause highly cytopathic infections of hepatocytes, stimulate much higher levels of NK/LGL in the liver (Table 27). This is also apparent in beige mutant mice which, despite having defective NK cell function (Roder and Duwe, 1977), respond to cytopathic MHV infection with large numbers of abnormal NK/LGL. In these circumstances, the presence of the virus, by virtue of its lysing target cells, may provide an important stimulus for NK cell accumulation as virus-infected cells have been shown to release factors which are chemotactic for leukocytes (Ward et al., 1972).

Bukowski et al (1985) demonstrated that NK cells play a role in the early resistance to MCMV infection, and suggested that NK cells may provide resistance against MHV infection as well (Bukowski et al., 1983b). In contrast, LCMV infection does not seem to be regulated by NK cells (Bukowski et al., 1985; Bukowski et al., 1983a,b; and present results). The tendency of a particular virus infection to attract a significant number of

NK/LGL to a site of virus replication may be one factor in determining its sensitivity to NK cell-mediated anti-viral resistance.

Several lines of evidence support the notion that the CTL lytic activity of liver leukocytes is being mediated by cells with an LGL morphology: 1) the CTL activity and the LGL present in the liver on day 7 p.i. are mostly resistant to *in vivo* treatment with anti-asialo GM₁, a treatment which depletes NK/LGL (Table 24); 2) leukocytes isolated from livers at day 7 p.i. with LGL morphology and CTL activity are at least partially sensitive to *in vitro* treatments with anti-Thy 1.2 + C and anti-Lyt-2 + C (Table 20); 3) CTL activity and LGL at day 7 p.i. are enriched in FACS-sorted Lyt-2⁺ leukocytes (Table 21), and 4) the levels of CTL activity and numbers of LGL are very closely correlated on days 7-14 p.i. (Figure 2). Thus, this suggests that virus-specific CTL in the liver at day 7 p.i. are LGL, and that the activity and number of these cells are directly related to the extent of virus replication that has occurred in this organ. The recent demonstrations that virus-specific CTL generated in the spleen acquire LGL morphology (Biron et al., 1986) and that adoptive transfer of virus-specific CTL can damage LCMV-infected hepatocytes *in vivo* (Zinkernagel et al., 1986) further support this claim.

The basis for the large inflammatory cell response in the livers of mice infected with hepatotropic LCMV-WE was likely to

be due either to an enhanced nonspecific inflammatory cell response which, by chance, included a proportion of CTL/LGL, or to virus-specific T cells homing to the LCMV-WE-infected liver and generating signals which attracted other inflammatory cells into the organ. The lack of a large LCMV-WE-induced inflammatory cell infiltrate in mice at 3 days postinfection (Figure 2) or in nu/nu mice at 7 days p.i. (Table 26) suggests that the T cell hypothesis may be correct. Supporting this hypothesis is that inflammatory responses in the brain or footpad of LCMV-infected mice are dependent upon the presence of H-2K- or D-restricted T cells (Doherty et al., 1976; Zinkernagel, 1976). The fact that a higher inflammatory response in the liver was seen with LCMV-WE than with LCMV-ARM is probably due to the fact that LCMV-WE replicates to higher titers (>100-fold) in the liver. LCMV-ARM infection did not appear to prevent accumulation of leukocytes in the liver, as mice co-infected with both LCMV-ARM and LCMV-WE accumulated numbers of liver leukocytes and LGL higher than mice infected with LCMV-ARM alone and these numbers were not significantly different than mice infected only with LCMV-WE (data not shown).

The majority of experimentation in murine viral immunology has relied on findings obtained predominantly from spleen cells. The data presented in Table 25 demonstrate that, under certain circumstances, cytotoxic activity mediated by spleen cells may not accurately reflect ongoing immune responses elsewhere in the

body. The basis for the markedly reduced spleen CTL response in C57BL/6J mice infected with LCMV-WE (compared to LCMV-ARM-infected mice) is not known. Two possibilities are that there is a defect in the spleen for the generation of LCMV-specific CTL under these conditions of LCMV infection, or else the liver may serve as a stimulus to rapidly recruit CTL out of the spleen. These findings underscore the importance of examining other compartments of the virus-infected host for signs of a localized immune response, especially if the virus exhibits specific tissue tropism(s).

Thus, it has been shown that during virus infection two waves of LGL influx occur in the liver, and they are associated with the histopathological features of viral hepatitis. The first of these is a thymus-independent population of NK/LGL which is significantly larger in cytopathic virus infections than in non-cytopathic virus infections. The second wave of LGL is a thymus-dependent influx occurring later during infection and containing Lyt-2⁺ CTL/LGL. Significantly greater numbers of CTL/LGL accumulate in the liver during an hepatotropic virus infection than during a non-hepatotropic virus infection.

C. Blastogenesis of LGL in the liver during viral infection

These studies document that the liver-associated increases in NK/LGL and CTL/LGL seen at early and late stages of viral infections, respectively, are associated with high levels of

blastogenesis within these populations. This contrasts with granulocyte responses, which are associated with accumulation of non-dividing, end-stage cells in pathological lesions. At one time NK cells were thought to be end-stage cells because of their relative resistance to irradiation (Kiessling et al., 1977; Hochman et al., 1978). This resistance, however, was a property of endogenous, unstimulated NK cells, few of which are blasts (Biron and Welsh, 1982). After IFN stimulation brought about by viral infection, the NK cell population converts to a rapidly dividing, blast cell-enriched population (Biron and Welsh, 1982; Biron et al., 1984). Other studies showed that lethal total body irradiation greatly reduces the NK/LGL infiltrate in the organs of virus-infected mice (McIntyre et al., 1987), confirming that cell division is required for the accumulation of these cells.

The kinetics of the NK/LGL blast response in the liver is similar to that of the spleen and parallels that of the IFN response. By mechanisms still unclear, IFN induces the blastogenesis and proliferation of NK cells in vivo (Biron et al., 1984). At late stages of the LCMV infection, a significant number of the Lyt-2⁻ LGL population mediating NK cell activity can still be found in the liver (Table 38). However, the IFN response at that time is low, and a very low percentage of the Lyt-2⁻ LGL have blast morphology. At this time there, nevertheless, are high levels of blastogenesis within the CTL (Lyt-2⁺ LGL) component (Table 38).

The presence of large numbers of blast NK/LGL and blast CTL/LGL in virus-infected livers raises the question as to their origin. Cell division could be taking place in these organs, and/or cells with blast morphology could be preferentially attracted to or trapped within this compartment. Data presented indicate that a significant proportion of NK cells in the virus-infected liver are ^3H -thymidine-incorporating effector cells (Table 35). This suggests that NK cells are, in fact, capable of at least one round of proliferation in non-lymphoid organs but does not rule out the possibility that many of the blast LGL may have recently migrated into the virus-infected organs from other compartments. Adoptive transfer experiments indicate that antigen-sensitized blast lymphocytes migrate into sites of inflammation more readily than do small lymphocytes (Asherson et al., 1973; Moore and Hall, 1973; McGregor and Logie, 1974). This preferential accumulation is apparently not antigen-specific (Asherson et al., 1973; Moore and Hall, 1973; McGregor and Logie, 1974), and may be at least partially explained by the blast cells' greatly increased motility (Hoffman et al., 1981). Furthermore, localized production of IFN may enhance the accumulation of migrating lymphocytes at sites of inflammation (Duijvestijn et al., 1986; Issekutz et al., 1986). Regardless, a preferential accumulation of the responding blast lymphocytes that are generated in vivo during a developing immune response may provide a means for the selective concentration of both non-

specific (NK/LGL) and antigen-specific (CTL/LGL) blast effector cells at the sites of antigenic stimulation.

It was hypothesized that the spleen would be a prominent source of NK/LGL for peripheral target organs, since mature NK cell blastogenesis and proliferation in the spleen increase dramatically during infection, while the lymph nodes, peripheral blood, and bone marrow contain very few functional proliferating NK cells (Biron and Welsh, 1982; Biron et al., 1984; Welsh et al., 1984a). However, neither splenectomy (Table 33) nor shielding the spleen (Table 32) or bone marrow (Table 31) during irradiation yielded any evidence implicating these sources as the major contributor of NK/LGL response in the liver. It is interesting to speculate that the liver itself may provide NK/LGL from its pre-formed pool of effector cells for NK/LGL responses elsewhere in the body.

CTL precursors proliferate in vivo in response to viral (Biron et al., 1986; Allen and Doherty, 1985) and allogeneic (Denizot et al., 1986) antigens, and they can differentiate into cytolytic cells at a site of localized antigenic stimulation (Ascher et al., 1981). At day 3 p.i. with LCMV the liver has few Lyt-2^+ cells, and they exhibit no virus-specific lytic activity (Table 38). By day 7 p.i., the proportion of Lyt-2^+ cells increases dramatically in this compartment and high percentages of the Lyt-2^+ LGL display blast cell morphology. The presence of these Lyt-2^+ blast LGL strongly suggests that CTL are also

capable of at least limited proliferation in this organ.

The kinetics of the LGL response varied with the viruses tested. Hepatotropic LCMV-WE infection induced the blastogenesis of a significantly greater proportion of the LGL in the liver than does non-hepatotropic LCMV-ARM infection. The extensive replication of LCMV-WE in the liver (Table 1; Welsh, 1984b) apparently serves as a strong stimulus for local LGL blastogenesis, which likely contributes to the greater numbers of total LGL (Figure 2) and blast LGL (Figure 6) at day 7 p.i. as compared to LCMV-ARM infection. In contrast, a virus-specific CTL response to MHV infection has not been described (Wege et al., 1982), and MHV does not induce the accumulation of large numbers of LGL in infected organs at day 7 p.i. (Natuk and Welsh, 1987). These observations are supported by a lack of LGL blastogenesis late in MHV infection (McIntyre et al., 1987) when CTL would otherwise be expected to be undergoing proliferation.

These data have demonstrated that dividing NK cells are present in livers of virus-infected mice. Increases in NK/LGL number are sensitive to irradiation and are strongly associated with increased proportions of LGL with blast cell morphology and with killing mediated by ^3H -thymidine incorporating lymphocytes. In viral infections (such as LCMV) which induce a potent CTL response, CTL/LGL blastogenesis also occurs in the liver, peaking later in infection. The blastogenesis and proliferation of cytotoxic effector cells at localized sites of viral replication

may provide a mechanism for in situ amplification of crucial components of the host's anti-viral defenses.

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