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The Role of Natural Killer Cells and Interferon in Virus Infections: A Thesis

Jack F. Bukowski
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THE ROLE OF NATURAL KILLER CELLS AND INTERFERON IN VIRUS INFECTIONS

A Thesis Presented

By

Jack F. Bukowski

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

August 1984

IMMUNOLOGY
TO CHA, MOM, AND THE MEMORY OF DAD
THE ROLE OF NATURAL KILLER CELLS AND INTERFERON IN VIRUS INFECTIONS

A Thesis

By

Jack F. Bukowski

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August 1984
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ABSTRACT

Jack F. Bukowski, Ph.D. Immunology, August 1984, University of Massachusetts Medical School.

Definitive evidence that natural killer (NK) cells mediate an antiviral effect in vivo was obtained using murine cytomegalovirus (MCMV) as a model system. Adoptive transfer studies using a variety of physical and immunochemical techniques to enrich and deplete NK cell activity showed that the cell population capable of mediating resistance (as assayed by enhanced survival and reduction in spleen virus titers) had the phenotype of an NK cell: nylon wool nonadherent, asialo GM1+, NK 1.2+, Ly 5+, Thy-1-, Ia-, low-density lymphocyte. Adoptive transfer of IL-2-dependent cloned NK cells (but not T cells) also provided resistance. NK cells did not provide resistance to lymphocytic choriomeningitis virus (LCMV).

Selective depletion of NK cell activity by injection of mice with antibody to anti-asialo GM1 lowered resistance to MCMV, mouse hepatitis virus, and vaccinia virus but not to LCMV. NK cell depletion resulted in up to 1000-fold increases in spleen and liver virus titers, correlating with more severe pathology in these organs. NK cells were found to have antiviral effects early (0-3 days) but not late (6-9 days) postinfection. NK cell depletion resulted in markedly increased MCMV-induced suppression of T cell function, which is probably responsible for the delayed clearance of virus seen in these mice. NK cell depletion resulted in increased virus synthesis during persistent MCMV infection, but had no effect...
on the course of persistent LCMV infection, despite elevated NK cell and interferon (IFN) levels found in these LCMV-infected mice. The reason why NK cells play a role against MCMV but not LCMV infection was not due to differences in NK cells induced by these 2 viruses, but more likely due to target cell susceptibility. IFN pretreatment of MCMV-infected cells failed to protect them against NK cell-mediated lysis, whereas uninfected and LCMV-infected cells were almost totally protected. These IFN-pretreated, LCMV-infected cells were not resistant to cell-mediated lysis in general, as this treatment increased their sensitivity to virus-specific T cell-mediated lysis by 2-3-fold. This enhanced sensitivity to lysis correlated with increased surface expression of H-2 antigens, but not viral antigens. In summary, these studies provide compelling evidence that NK cells can mediate antiviral effects in vivo, and provide some insights into their mode of action and consequences of their disfunction.
Abbreviations

NK  natural killer
IFN  interferon
ADCC  antibody-dependent cell-mediated cytotoxicity
MCMV  murine cytomegalovirus
MHV  mouse hepatitis virus
VV  vaccinia virus
LCMV  lymphocytic choriomeningitis virus
HSV-1  herpes simplex virus type 1
IL-2  interleukin 2
C'  complement
CPE  cytopathic effect
poly I:C  polyinosinic:cytidylic acid
CTL  cytotoxic T lymphocyte
i.v  intravenous
i.p.  intraperitoneal
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CHAPTER 1

Introduction

Characteristics of Natural Killer (NK) Cells

Natural killer (NK) cells (1) are medium to large granular lymphocytes (2) possessing the capability to lyse certain types of target cells both in vitro (1) and in vivo (3). They have been found in every mammalian species examined so far, including man (4), mouse (5), hamster (6), and rat (7). NK cells do not require prior exposure to target cells in order for lysis to occur and do not exhibit immunologic memory (1). The basis of NK cell recognition of target cells is unknown, but is the subject of intense investigation in many laboratories. NK cells do not adhere to plastic or to nylon wool (5), which distinguishes them from macrophages. They do not bear surface immunoglobulin (8), as do B cells, nor do they possess the T cell antigens Ly 1 or Ly 2 (9). They possess low quantities of surface thy-1 antigen (10), but are present in T cell-deficient athymic nude mice (11), indicating that they are distinct from classically defined T cells. They bear Fc receptors for IgG (12), and have the ability to recognize and destroy antibody-coated targets by binding the Fc portion of IgG (13). NK cells bear high quantities of the neutral glycosphingolipid asialo GM1 on their surfaces (14), as well as NK alloantigens (15).

In the mouse, NK cells are present at high levels in the
peripheral blood and spleen, and at lower levels in the bone marrow, lymph nodes, lungs, and peritoneum; NK cell activity is absent in the thymus (1,5). Newborn mice have low NK cell activity until 4 weeks of age. Peak NK cell activity occurs at 4-10 weeks of age, and declines thereafter in the spleen (1,5), but less markedly in the peripheral blood (16).

Because of their apparent nonspecificity and their ability to efficiently lyse tumor cells on contact without prior sensitization, NK cells may represent a first line of defense against tumor cells. The evidence for a role of NK cells in mediating tumor surveillance now overwhelmingly supports the concept. Early evidence demonstrated a correlation between high NK cell activity and tumor resistance. Mice whose NK cell activity is low due to genetic predisposition, age, nonspecific immunosuppression, or the beige mutation have deficiencies in resisting tumor implants and controlling metastases (17-19). In syngeneic systems, there is a good correlation between in vitro sensitivity of tumor cells to lysis by NK cells and their ability to be rejected in vivo (17,19). More recent evidence showing that selective depletion of NK cell activity by injection of antibody to asialo GM1 (20) or to NK 1 (21) results in increased tumor growth and decreased clearance of tumor cells considerably strengthened the hypothesis that NK cells had an antitumor role. Definitive evidence for this hypothesis was obtained in adoptive transfer studies when NK cell-depleted mice reconstituted with NK cell-containing spleen cells resisted tumor growth while those given NK cell-depleted spleen cells did not.
Despite progress made in assessing the role of NK cells in tumor surveillance, the role of NK cells in virus infections has not been definitively determined until now, and is the subject of this thesis.

Characteristics of Interferon (IFN)

The interferons are a group of glycoproteins first described by Isaacs and Lindenmann as having the ability to protect cells against virus infections (23). There are 3 major types of interferon (IFN), but each of these is presently being divided into subtypes based on DNA sequence and biological function using homogeneous preparations of cloned IFN's. The 3 major subtypes have so far been shown to behave similarly in biological assays and are classified as follows (reviewed in 24): type alpha is produced by leukocytes in response to stimulation with viruses or polynucleotides, whereas type beta is produced by fibroblasts and epithelial cells in response to these same stimulators. Type gamma is produced by lymphocytes undergoing immune stimulation by antigen or stimulation by plant lectins.

Biological effects mediated by IFN have been described extensively (reviewed in 24), but will be dealt with more thoroughly in a subsequent section.

NK Cells as Mediators of Host Resistance to Virus Infections--
an Historical Perspective
In 1977 a number of investigators (25-28) reported that virus infections in mice rapidly augmented the lytic activity of non-specific lymphocyte effector cells termed "natural killer" cells by Kiessling et al. (1). These activated NK cells have increased target cell range (25,29), enhanced kinetics of lysis (30), and an ability to kill over and over again (31). It was noted that NK cell activation closely paralleled the appearance of virus-induced IFN (32), and subsequent experiments demonstrated that IFN by itself was capable of activating NK cells (32,33) and causing their blastogenesis (34). These observations led to speculation that NK cells may be playing a role in natural resistance to virus infections, constituting a first line of defense, several days before antigen-specific T cells and B cells are mobilized. A large body of circumstantial evidence for this hypothesis exists in several virus systems, but until now, no one has been able to convincingly demonstrate an antiviral role for NK cells.

Several lines of evidence support a role for NK cells in resistance to virus infections. Strains of mice with genetically high NK cell activity have increased resistance to infection with certain viruses such as herpes simplex type 1 (HSV-1) (35), and murine cytomegalovirus (MCMV) (36) as compared to mice with genetically low NK cell activity. Nonspecific immunosuppression by cyclophosphamide and cortisone results in increased severity of HSV-1 (35) and MCMV (37) infections before virus-specific T and B cells are detectable in control, virus-infected mice. Newborn mice,
whose NK cell activity is low, are susceptible to MCMV (38, and mouse hepatitis virus (MHV) (39,40) until 3-4 weeks of age, at which time NK cell activity matures (1,5). In some virus systems, NK cells can lyse virus-infected cells more efficiently than uninfected cells (35,41). Each of these lines of evidence will now be discussed in detail.

Kumar and Bennett (42) noted a correlation between resistance to Friend leukemia virus (FLV) and resistance to bone marrow allografts. The latter type of resistance was first described by Cudkowicz et al. (43,44), who demonstrated that the cell responsible for the rejection of bone marrow allografts was itself dependent on an intact bone marrow environment, and was genetically determined by two dominant loci outside the H-2 complex. This system of resistance, termed the hematopoietic histocompatibility (Hh) system, was mediated by a type of lymphocyte termed a marrow-dependent (M) cell, which bore no similarity to classically defined T cells. Meanwhile, Kiessling et al examined the inheritance pattern of NK cell activity in mice, and found that this pattern was similar to that described for the Hh system (45), indicating that the M cell and the NK cell may be the same, and since strains with genetically high NK cell activity are more resistant to FLV infections than those with low NK cell activity (42), the evidence, though only correlative, suggests that NK cells may be mediating resistance to FLV. Similar evidence linking the ability to reject bone marrow allografts with resistance to infection was obtained by Lopez (35) using HSV-1.
Another line of evidence which correlates the genetics of resistance to virus infections with the genetics of NK cell activity is the work of Shellam and his colleagues in the (MCMV) system (36). Mice with genetically high NK cell activity were more resistant to the lethal effects of MCMV than mice with genetically low NK cell activity. Further work indicated that mice homozygous for the beige mutation (bg/bg), a condition resulting in low NK cell activity, were more susceptible to MCMV than heterozygous (bg/+) phenotypically normal mice with high NK cell activity (46). Transfer of bone marrow cells from bg/+ donors to bg/bg recipients conferred resistance upon the recipients challenged 8 weeks later with MCMV, indicating that resistance was mediated by a bone marrow-derived cell from bg/+ mice. These data indicated a good correlation between the presence of high NK cell activity and resistance to MCMV. However, the beige mutation, though more selective in its immunosuppressive effects than cortisone and cyclophosphamide treatments, is not completely selective for NK cell function, as other immune functions such as neutrophil and platelet functions are abnormal (47). Because several immune functions are affected by the beige mutation, it was not certain which one was responsible for the resistance against MCMV.

Another piece of circumstantial evidence implicating NK cells as mediators of resistance to virus infections is the time frame during which animals become sick. Adult mice given large doses of MCMV (46) or HSV-1 (35) exhibit marked genetic differences in susceptibility within 2 days of inoculation, and susceptible mice
die between 3 and 7 days postinfection. Two days postinfection falls before that time during which specific immune responses such as virus-specific cytotoxic T cells (CTL) (48) and antibodies (49) are detectable. Even more suggestive is the fact that newborn mice suddenly acquire resistance to MCMV (39) and (MHV) (40) between 3 and 4 weeks of age, correlating with the development of NK cell activity (1,5).

There is also a body of evidence which suggests that NK cells do not play a role against virus infections such as Sindbis and LCMV (50,51), despite the ability of these viruses to activate NK cells in vivo (32,51). NK cell-deficient beige mice did not exhibit greater sensitivity to LCMV (50), or Sindbis (51) infection than control mice. Radiation or cyclophosphamide treatments did not increase virus titers in mice persistently infected with LCMV (52). There is no indication that genetically high NK cell activity confers upon mice resistance to LCMV (53).

One potential mechanism whereby NK cells could mediate antiviral effects is by preferential lysis of virus-infected cells. There are several ways this can happen. Santoli et al. (54,55) showed that increased in vitro NK cell-mediated lysis of influenza-and HSV-1-infected cells can occur as a result of IFN induction and subsequent activation of endogenous NK cells during 16 hour cytotoxicity assays, but not during shorter assays. This has also been shown to occur in the mouse system (56). However, enhanced lysis of virus-infected cells by endogenous NK cells in culture is not always due to IFN, as Lee and Keller (41) demonstrated that
MCMV-infected fibroblasts are killed by endogenous spleen cells more efficiently than uninfected fibroblasts in the absence of detectable IFN. MCMV-infected fibroblasts are innately less sensitive than uninfected fibroblasts to lysis by preactivated NK cells (36), so perhaps the endogenous NK cells in culture are being activated by an IFN-independent mechanism. Viral glycoproteins from mumps (57) and measles (58) viruses have been shown to activate human NK cells independent of IFN. This activation could lead to enhanced lysis of virus-infected cells. Enhanced binding of NK cells to virus-infected targets has been reported in the human system with Epstein-Barr virus (59), and in the mouse system with Sendai virus (56), but this enhanced binding does not always lead to enhanced lysis, as Sendai-infected L-929 cells are actually more resistant to NK cell-mediated lysis despite the fact that they bind NK cells better (56).

Virus infection may somehow increase the innate sensitivity of a target cell to NK cell-mediated lysis, but this has not been convincingly demonstrated (60). More often it is observed that virus infection actually decreases the susceptibility of target cells to lysis by previously activated NK cells. This has been seen with MCMV (36), Sendai, Sindbis, and vesicular stomatitis virus (VSV) infections (56). HSV-1-infected Vero cells do not bind NK cells and therefore resist lysis (56), but the mechanism of decreased resistance to lysis in the other viral systems is not known, but could involve protection of these cells by IFN (discussed below), or a reduced ability of these cells to trigger the NK cell
lytic mechanism.

Trinchieri et al. (61) showed that IFN treatment of target cells can influence their sensitivity to NK cell-mediated lysis, depending on the condition of the target cell. Whereas a variety of normal target cells are protected by IFN against NK cell-mediated lysis, target cells whose metabolic functions have been altered by treatment with actinomycin D or cyclohexamide are no longer IFN-protected. Cells infected with influenza or vaccinia virus (VV) are not protected (61). Thus, IFN can protect normal cells from NK cell-mediated lysis, while leaving virus-infected cells susceptible to lysis. Welsh and coworkers (62) have shown that IFN-mediated protection of target cells occurs in vivo, but it is still not clear what role, if any, this mechanism plays in resistance to virus infections.

Definitive evidence that NK cells are or are not playing a role against virus infections has been lacking. Further, the evidence for or against an antiviral role for NK cells has often been conflicting. For example, LCMV (32), Sindbis (51), MCMV (36) and HSV-1 (35) are all capable of activating NK cells in vivo, but genetic or drug-induced deficiencies in NK cell activity correlate with lowered resistance to MCMV (46) and HSV-1 (35), but not LCMV (50), or Sindbis (51) virus infections. Activated NK cells lyse HSV-1 (56) and MCMV-infected (36) targets less efficiently than uninfected, Sindbis virus-infected, or LCMV-infected cells (56), making it difficult to attribute in vivo resistance to MCMV and HSV-1 to NK cell-mediated lysis as assayed in vitro. One of the
aims of this thesis is to clarify these data by accurately determining which virus infections are subject to NK cell-mediated resistance, and by using these data to determine how NK cells mediate resistance, and why they may be effective in controlling some virus infections, but not others.

The Role of IFN in Virus Infections:
Mechanisms of Antiviral Action In Vivo

The protective role of IFN induced during virus infections has been clearly demonstrated. Fauconnier (63) injected mice with antibody to IFN and observed that Semliki Forest virus infections in these mice produced greater mortality in these mice as compared to control virus-infected mice. Gresser et al. (64,65) showed that antibody to IFN enhanced virus replication, pathogenesis, and mortality in mice infected with HSV-1, VSV, encephalomyocarditis, Moloney sarcoma, and Newcastle disease viruses. However, these investigators showed that anti-IFN had no effect on intranasal influenza virus infection, although it did exacerbate intranasal VSV infection (65).

Whereas the above studies show that endogenous IFN provides resistance to viral infections, definitive evidence regarding its mode of action in vivo is lacking. There are a number of ways in which IFN could be mediating antiviral effects, but most of this evidence has been gathered in in vitro systems. IFN could protect by simply bathing an area of susceptible cells and inhibiting virus
replication (23). Alternatively, it could serve to potentiate cell-mediated defense mechanisms, such as NK cells. IFN induces activation (32,33) and blastogenesis (34) of NK cells, and could thus potentiate NK cell-mediated destruction of virus-infected cells. IFN enhances the phagocytic (66) and cytotoxic (67) functions of macrophages, cells which might have antiviral effects (68,69,70). IFN may enhance antibody production (71) and may be required for the generation of CTL (72), which may be responsible for clearance of virus in vivo (73).

There have also been studies demonstrating the in vivo efficacy of treatment with exogenous IFN or inducers. In general, these treatments are most efficacious when administered early in the infection or before infection (24). The first demonstration that IFN could mediate antiviral effects in vivo was by Lindenmann et al. (74), who showed that prophylactic treatment with IFN protected rabbit skin against vaccinia virus. Prophylactic IFN is also effective against a generalized infection, as Denys (75) showed that IFN could protect rats against generalized Sindbis virus infection. The IFN inducer poly I:C was effective in protecting against HSV-1 keratitis infection in the eyes of rabbits (76). Again, the mode of protection by IFN in these studies was not determined, but in vitro evidence (see above) suggest possible mechanisms. The data in this thesis provide new in vivo evidence pertinent to the in vivo mode of antiviral action of IFN, and provides new in vitro evidence suggesting mechanisms whereby IFN may mediate antiviral effects in vivo.
Aim of the Thesis

The aim of this thesis is to present data addressing the following questions:

1. Do NK cells provide resistance against acute and persistent virus infections?
2. What are the pathological consequences of NK cell depletion during acute virus infections?
3. By what mechanisms do NK cells mediate antiviral effects?
4. In what ways does IFN enhance the ability of host defenses to mediate antiviral effects?
CHAPTER 2

Manuscripts

Throughout the discussion section (Chapter 3), manuscripts will be referred to by their Roman numerals (I–VI). Tables and Figures within these manuscripts will be referred to by placing a notation after the relevant manuscript. For instance, Table 3 in manuscript number II will be written as: II-Table 3.

This thesis is based on the following manuscripts:


natural killer cells in vivo. Submitted to J. Exp. Med.

Elevated Natural Killer Cell-Mediated Cytotoxicity, Plasma Interferon, and Tumor Cell Rejection in Mice Persistently Infected with Lymphocytic Choriomeningitis Virus
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Footnotes

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2. Abbreviations Used

125IUDR - 5-iododeoxyuridine
LCMV - lymphocytic choriomeningitis virus
VSV - vesicular stomatitis virus
SFV - Semliki Forest virus
IFN - interferon
Asialo GM₁ - gangliotetraosylceramide
C¹ - complement
HSV - herpes simplex virus
NK - natural killer
NDV - Newcastle disease virus
MEM - minimal essential medium
CPE - cytopathic effect
poly I:C - polyinosinic:cytidylic acid
FBS - fetal bovine serum
S.D. - standard deviation

Abstract

To assess the effects of chronic virus infection on natural killer (NK) cells, the related phenomena of interferon (IFN) production, NK cell activation, and resistance to tumor implants were studied in mice persistently infected with lymphocytic choriomeningitis virus (LCMV). NK cells from these LCMV-carrier mice displayed augmented killing of the NK-sensitive YAC-1 target cell. They did not lyse the more resistant targets L-929 and P815, while NK cells from acutely infected mice efficiently lysed all three cell types. The plasma from LCMV-carrier mice contained an antiviral substance identified as IFN type I, based on species specificity, virus non-specificity, resistance to pH 2, and sensitivity to antibody to type I IFN. IFN titers in plasma from LCMV-carrier mice were 32 to 64 U/ml, about 20 - fold less than those in acutely-infected mice. Both the IFN and NK cell levels continuously remained elevated in the LCMV carrier mice up to at least 6 months of age. IFN is known to activate NK cells and to induce their blastogenesis in vivo. As determined by centrifugal elutriation, large NK blast-size cells were isolated from the spleens of acutely infected mice, but not from either normal or LCMV-carrier mice, suggesting augmented NK cell-mediated lysis in the absence of enhanced proliferation. Polyinosinic-cytidylic acid induced high levels of NK cell-mediated cytotoxicity and blastogenesis in both control and LCMV-carrier mice, but IFN was induced to lower levels in carriers as compared to controls. Coincidental with augmented NK cell activity, the LCMV-carrier mice rejected intravenously injected $^{125}$IUDR-labeled
tumor cells more efficiently than did normal mice. Thus, LCMV carrier mice have low levels of type I IFN, moderately augmented NK cell activity lasting for at least 6 months, and increased resistance to tumor cell implants. This indicates that augmented NK cell-mediated cytotoxicity can be maintained in vivo over prolonged periods of time in the presence of chronic low-level IFN stimulation.
Introduction

It has been known for several years that interferon (IFN) induced during acute viral infection activates natural killer (NK) cells (1,2). A substantial body of evidence now indicates that NK cells play a role in tumor surveillance (reviewed in 3). Talmadge et. al. (4) have shown that mice whose NK cells have been activated by acute viral infection are more resistant to tumor growth and metastasis than are uninfected mice. However, very little is known about the effect of persistent viral infection on NK cells and tumor resistance.

A well characterized persistent infection is that of lymphocytic choriomeningitis virus (LCMV) in mice. Mice infected in utero or at birth carry this Arenavirus for life and often do not show overt disease symptoms (5), though they do suffer from chronic immune complex disease (6). These LCMV-carrier mice have LCMV-specific antibody existing in the form of immune complexes (7), but they have no detectable cytotoxic T cells specific for LCMV and they respond abnormally to certain non-viral antigens (8). Using this model we examine the three interrelated phenomena of IFN production, NK cell activation, and tumor resistance during chronic virus infection.

We report here that 1) LCMV-carrier mice have levels of NK cell-mediated cytotoxicity intermediate between normal and acutely infected mice, 2) these carriers have low but detectable levels of plasma IFN, and 3) these carriers reject tumor cells more efficiently than do normal mice.
Materials and Methods

Animals. BALB/c byJ mice were purchased from Jackson Laboratories, Bar Harbor, ME. SWR/J mice were kindly provided by Dr. Aldo Rossini, UMass Medical School. (BALB/c byJ X SWR/J) F1 mice were bred in our own facilities.

Cells. YAC-1 cells were derived from a Moloney leukemia virus-induced lymphoma in A/Sn mice. P815 cells were from a methylcholanthrene-induced mastocytoma in DBA/2 mice. These cells were maintained in RPMI-1640 medium (GIBCO, Grand Island, NY) supplemented with antibiotics, glutamine and 10% heat-inactivated fetal bovine serum (FBS) (M.A. Bioproducts, Walkerville, MD). Vero cells are a continuous monkey kidney line, and L-929 cells are a continuous liver cell line from C3H mice. These cells were maintained in minimal essential medium (MEM) with the same additives as listed above.

Virus. The lymphocytic choriomeningitis virus (LCMV) used in these studies was the Armstrong strain (9). The Indiana strain of vesicular stomatitis virus (VSV) was obtained from Dr. John Holland, UCSD, San Diego, CA. Semliki Forest virus (SFV), neurotropic strain, and herpes simplex virus type I (HSV-I), a mouse-adapted strain, were obtained from Dr. Michael Oldstone, Scripps Clinic, San Diego, CA.

Antisera. Rabbit anti-asialo GM₁, a gift from Drs. S. Habu and K. Okumura (10), was used in vitro at a final dilution of 1:150 and in vivo by injecting mice intravenously with 200 ul of a 1:40 dilution.
The antiserum to NK 1.2 was a gift from Dr. Robert Burton (11) and was used at a final dilution of 1:40. Monoclonal anti-thy 1.2 was provided by Dr. Edward Clark, Genetic Systems Corp., Seattle, WA and used at a final dilution of 1:50. For in vitro treatment of cells, 1 X 10^7 spleen leukocytes suspended in 100 ul serum-free medium were treated with 50 ul antiserum for 30 min at RT. Twenty-five ul complement (C') were then added, and the mixture was further incubated at 37°C for 45 min. Guinea pig serum absorbed with spleen leukocytes was the source of C'. Rabbit serum was used as the source of C' with anti-NK 1.2. The cells were then pelleted and resuspended in assay medium. Antiserum to IFN-α/β (type I) was obtained from the NIH and used at a final dilution of 1:100.

Interferon Assay. Blood was collected in heparinized Natelson tubes from the retro-orbital sinus of mice anaesthetized with ether. Plasma was obtained by centrifugation and was titrated by 2-fold serial dilutions in a 96 well flat bottom microtiter plate. L-929 or Vero cells were then added at 3 x 10^4 cells/well. Eighteen to twenty-four hours later, the wells were challenged with 100 TCID_{50} units of VSV. SFV and HSV-1 were also used to assay for virus specificity. IFN titers were expressed as the log_{2} of the highest reciprocal dilution resulting in a 50% reduction in CPE.

Interferon. IFN type I (β) was purchased from Lee Biomolecular (San Diego, CA). IFN type II (γ) was from the 48 hour supernatant of 10^7 mouse spleen cells incubated in 10 ml of RPMI with 2 ug/ml concanavalin A (Sigma, St. Louis, MO).
Establishment of LCMV-Carrier Mice. BALB/c mice were injected intraperitoneally with 0.03 ml LCMV at 8 \times 10^4 pfu/ml within 24 hours of birth (5). In some animals, 3 X plaque-purified LCMV was used as an inoculum. Congenital carriers, infected in utero by the mother's persistent infection, were maintained in our facilities. Experiments were done with congenitally infected mice, unless otherwise stated.

Cytotoxicity Assay. Assay medium was RPMI supplemented with 0.01 M N-2-hydroxylethyl piperazine-N\(^1\)-2-ethane sulfonic acid (HEPES) (Sigma), 10% fetal bovine serum, glutamine, and antibiotics. The assay was performed as described (1). Briefly, target cells labeled with 100 uCi sodium chromate (\(^{51}\)Cr; Amersham Corp., Arlington, Hts., IL) for 1 hour at 37°C were washed and mixed with effector cells in round-bottom microtiter wells at 10^4 target cells/well. For spontaneous release determination, medium was added to the wells and 1% Nonidet P-40 was added for maximum release determination. Plates were incubated for 6 to 16 hours at 37°C in a humidified atmosphere of 5% CO\(_2\), 95% air. At the end of incubation, plates were centrifuged at 200 g for 5 min., and 0.1 ml of the supernatant was pipetted off and counted in a Beckman gamma 5500 counter (Beckman, Palo Alto, CA). Data are expressed as % specific release:

\[
100 \times \frac{\text{cpm experimental} - \text{cpm spontaneous}}{\text{cpm maximum} - \text{cpm spontaneous}}
\]

Centrifugal Elutriation. Size separation of cells was accomplished using a Beckman JE-6B centrifuge. About 3 X 10^8 spleen leukocytes
were treated with deoxyribonuclease (Sigma) to prevent clumping. These cells were loaded into the rotor which was spinning at 3200 rpm at 5°C. The rate of flow of the elution medium (Hank's balanced salt solution, 1.5% calf serum) was 15, 22, 28, 32, 38 and 46 ml/min and corresponded to fractions 1-6, respectively. Cell recovery was about 65%.

Poly I:C Induction. Some mice were injected intraperitoneally with 100 ug polyinosinic:cytidylic acid (poly I:C) (Sigma) dissolved in phosphate-buffered saline. IFN titers and NK activities were determined 18-24 hr post-injection (12).

In Vivo Rejection Assay. In this procedure described by Riccardi et. al. (13), 50 ml of YAC-1 cells in the log phase of growth at 2 X 10^5/ml were labeled with 20 uCi ^{125}IUDR in an upright 75 cm^2 flask for 18 hours at 37°C. When L-929 cells were used, 1.5 X 10^6 cells in 25 ml MEM were seeded into a 75 cm^2 flask, incubated for 2 days, and labeled with 20 uCi ^{125}IUDR for 18 hr prior to harvest. After extensive washing, the cells were adjusted to 2 X 10^6 cells/ml for YAC-1 and 1 X 10^6 cells/ml for L-929 and 0.5 ml of the suspension was injected i.v. into each recipient mouse. Two to four hours later, the mice were sacrificed, their spleens were assayed for NK activity, and their lungs were placed into test tubes and counted for radioactivity in a gamma counter.
Results

NK Cell Levels of LCMV-Carrier Mice. When compared to age-matched controls, LCMV-carrier mice had significantly higher cytotoxic activity against YAC-1 targets (Table 1). This was seen both in short (6 hr) assays where little activity at all was mediated by normal cells (Table 1A), and in long (16 hr) assays, which demonstrated that the low endogenous NK strain, BALB/c, had splenocytes capable of killing YAC-1 cells to a significant degree (Table 1B). Endogenous NK cell activity in mice declines rapidly after 12 weeks of age, even in strains with genetically high activity (1). However, even at 26 weeks of age, spleen cells from LCMV carrier mice continue to lyse YAC-1 targets very efficiently, (Table 1A).

Table 2 shows that LCMV-carrier cytotoxic cells are intermediate in activity between endogenous NK cells and those activated by acute LCMV infection. Carrier cells lyse YAC-1 targets more efficiently than do normal cells, but unlike NK cells from acutely infected mice, they do not efficiently lyse NK-insensitive L-929 cells (Table 2) or P815 cells (data not shown).

Characteristics of Splenic Cytotoxic Cells in Carrier Mice. To further test the hypothesis that the cytotoxic cells were in fact NK cells, they were subjected to treatment with various antisera and complement in vitro. Table 3 shows the results of these experiments. When treated with antisera to the NK cell antigens NK 1.2 or asialo GM₁ and C', leukocyte cytotoxicity against YAC-1 cells was drastically reduced.
Treatment of carrier mice with anti-asialo GM$_1$ in vivo also depleted the cytotoxic activity. In contrast, very little cytotoxic activity was deleted when monoclonal anti-thy 1.2 antibody and C' was used. Similar results were obtained when these reagents were used to deplete NK cell activity in control or three day-acutely infected BALB/c mice. Untreated or C' treated control spleen leukocytes mediated 16 to 18% lysis (at 100:1 effector to target ratios in a 14 H assay), while 0.4, 2.9 and 19% lysis was observed after treatment with anti-asialo GM$_1$, anti-NK, and anti-thy 1.2 antibodies, respectively. Untreated or C'-treated acutely infected spleen leukocytes lysed 68 to 74% of targets (at 33:1) whereas 6.8, 39 and 70% lysis was mediated by cells treated with anti-asialo GM$_1$, anti-NK and anti-thy 1.2 antibodies, respectively. These studies thus provide strong evidence that the effector cells in the carrier mice are classically defined NK cells.

**Interferon Levels in Carrier Mice** Since NK cells are known to be activated by IFN (1,2), we performed IFN assays on spleen extracts and plasma from carrier and normal mice. Spleen extracts had no detectable activity (data not shown) but plasma from carrier mice had a low but significant level of IFN-like activity (32-64 units/ml; Table 4). Every carrier mouse examined (40 BALB/c, 12 SWR, and 9 (BALB/c x SWR) F1) had plasma IFN-like activity (>16 units/ml). In contrast, of the normal mice examined, only 4 of 21 BALB/c, 4 of 10 SWR, and 2 of 10 (BALB/c x SWR) F1 had >4 units/ml IFN. Acutely infected mice had substantially higher levels of IFN-like activity (Table 4), confirming previous reports (1).
In order to minimize the possibility that the IFN-like activity in the plasma of these carriers was brought about by an unknown contaminant of our LCMV stock, carriers were established by injecting newborn BALB/c mice with 3 x plaque-purified LCMV. Three of these mice were tested for plasma IFN at 5 weeks of age, and all three had 32 units/ml. Thus, LCMV was most likely responsible for the putative IFN production in the carriers.

Characteristics of Anti-Viral Substance in Carrier Mice. Since many investigators (14-17) have failed to find IFN in LCMV carrier mice, it was important to characterize the anti-viral substance we had tentatively called IFN. Carrier plasma did not induce anti-viral activity in Vero cells (data not shown) but did protect mouse L-929 cells from HSV-I, SFV (data not shown) and our standard assay virus, VSV. These experiments showed that the anti-viral activity was species specific and virus non-specific. Further, the antiviral activity in the carrier plasma was acid stable, but sensitive to treatment with antibody to type I IFN, consistent with the activity being type I and not type II IFN (Table 5). Since this antibody neutralizes both \( \alpha \) and \( \beta \) IFN, the distinction between them was not made. The acid stability of the antiviral activity also indicates that LCMV, an acid-labile virus, is not inducing IFN in vitro.

Augmentation of Cytotoxicity and IFN Levels With Poly I:C. To explore the effects of IFN on carrier splenic effector cells, these mice and age-matched controls were treated with poly I:C. Cytotoxicity mediated by cells from both normal and carrier mice was significantly elevated
**Tumor Cell Rejection In Vivo.** Because we had found that LCMV carriers had IFN and elevated NK activity, we questioned whether these carriers would reject tumor cells more efficiently than normal mice in a short term *in vivo* assay. *In vivo* rejection of $^{125}$IUDR-labeled tumor cells correlates with *in vitro* NK cell activity and with resistance to outgrowth of tumor cell implants (4,19). Table 8 shows that carrier mice are more efficient in clearing $^{125}$IUDR-labeled YAC-1 and L-929 cells from their lungs than normal mice. This increased rejection correlated with increased *in vitro* cytotoxicity against YAC-1 cells in a $^{51}$CR release assay (Table 8). Since L-929 cells are cleared differently by carriers and normal mice *in vivo*, carrier mice may be more active than normal mice in eliminating relatively NK-insensitive as well as sensitive targets.
by this treatment (Table 6), furthering our notion that the carrier effector cells were NK cells. Plasma IFN levels were elevated by poly I:C treatment in both carriers and normal mice (Table 7), but carriers had lower levels than normals, an observation consistent with previous work (14).

Size of Carrier NK Cells. Recent work in our laboratory (18) has shown that acute LCMV infection causes spleen NK cell blastogenesis in vivo. These blast NK cells are larger than those in uninfected mice and can be separated on the basis of size by centrifugal elutriation. To examine the size of carrier NK cells compared to acutely infected and uninfected mouse NK cells, these three types of spleen cells were subjected to elutriation, and each fraction was assayed for cytotoxic activity against YAC-1 targets. As shown before (18), acute LCMV infection caused a shift in peak NK activity from the smaller fractions to the larger ones (Fig. 1). In contrast, carrier NK cells, though exhibiting higher activity in each fraction, did not show a shift of peak activity to large, blast-size fractions. Thus, in contrast to the acute infection, there was no evidence for large-scale blastogenesis of NK cells in LCMV-carrier mice. Carrier NK cells were capable of undergoing blastogenesis if stimulated with poly I:C. Figure 2 shows that poly I:C treatment of LCMV-carrier or control mice results in very similar elutriation profiles, suggesting that carrier NK cells can undergo blastogenesis to the same extent as control NK cells.
Discussion

The results presented here demonstrate that mice persistently infected with LCMV have plasma IFN, elevated NK cell-mediated cytotoxicity, and are more efficient in rejecting tumor cells than normal mice. Our observation that LCMV-carrier mice have elevated NK cell-mediated cytotoxicity led us to a search for IFN in these mice despite the failure of others to detect it (14-17). To be sure that we were detecting IFN and not another antiviral entity (20), we characterized the substance and found that the activity was species specific, virus non-specific, acid stable, and sensitive to treatment with an antibody to IFN type I. It had previously been difficult to understand why LCMV-carrier mice did not produce IFN while acutely infected mice made high levels of IFN. We now know from our present results that carrier mice do make IFN, though at low levels. It should be noted that during the course of our present study, Saron et. al. (21) reported low levels of IFN in the serum of LCMV-carrier mice and demonstrated the IFN-induced enzyme 2' - 5' A synthetase in cells from these mice. It has been suggested (22) that the plaque reduction IFN assay used in the negative studies by others is not as sensitive as the assay employed by us and by Saron et. al. (21). Other possible reasons for discrepancies in results include virus and mouse strain differences. IFN induction was unlikely due to a contaminant in the virus preparation, as 3 X plaque-purified LCMV also induced IFN in carrier mice.
With the knowledge that LCMV-carrier mice have IFN, one might have predicted the outcome of the experiments in which poly I:C induced a lower level of IFN in carriers than in normal mice. It has been known for some time that cells stimulated to produce IFN are refractory (hyporesponsive) to a second stimulus for several days, i.e., they will produce less IFN than previously unstimulated cells (23). Holterman and Havell (14) observed that LCMV-carrier mice challenged with Newcastle disease virus (NDV) had lower serum IFN titers than NDV-challenged normals. However, this result could have been explained by the fact that LCMV-infected cells are resistant to superinfection with NDV (23). It now seems likely that hyporesponsiveness to IFN stimulators could account for this result. Saron et al. (25) reported that carrier mice produce more IFN after poly I:C inoculation, and concluded that their mice might be interferon primed. Both priming and hyporesponsiveness can be induced by IFN, but it is not known why the report by Saron et al. (25) conflicts with ours and that of others (14).

Another observation consistent with the presence of IFN in LCMV-carriers is the presence of elevated cytotoxicity against YAC-1 targets. This cytotoxicity was sensitive to treatment with anti-asialo GM₁ in vivo, anti-NK 1.2 or anti-asialo GM₁ and complement in vitro, but not to anti-thy 1.2 and complement and thus was mediated by NK cells. Previous studies failed to show NK cell activation in LCMV-carrier mice (1,26). However, one such study only looked at the lysis of L-929 cells, and we have shown here that the NK cells in the carrier
mice are not sufficiently activated to lyse those cells, in agreement with that previous result (1). In another limited study, no differences were observed between control and LCMV-carrier SWR/J mice, but extensive investigations on our part have shown that control SWR/J (but not BALB/c) mice sometimes have elevated NK cell mediated lysis comparable to the carriers. The reason for this is not known but could involve an undefined infection.

A series of experiments shown here using 6-month old mice revealed that cells from these LCMV-carriers had augmented NK cell mediated lysis and that the substantial differences between normals and carriers were apparent. Normal mice have peak NK cell levels at 4-10 weeks and by 6 months have little if any endogenous NK cell activity, but retain the ability to respond to IFN or IFN inducers (1). With LCMV-carriers, it is likely that the sustained presence of IFN in the plasma is maintaining the high NK cell levels.

Recent work in our laboratory has demonstrated that IFN induces blastogenesis of NK cells in vivo. Blast NK cells are also seen when high levels of IFN are generated during acute infection with LCMV (18). Although low levels of IFN were sustained in persistently infected mice (Table 4), large-size, blast-NK cells were not induced to a significant degree; NK cells isolated from LCMV-carrier mice were contained predominantly in small-size classes (Fig. 1). NK cells from carrier mice were, nevertheless, capable of blastogenesis, since poly I:C treatment induced higher levels of IFN and blast-NK cells (Fig. 2). The elevated lysis mediated by NK cell populations isolated from
carrier mice could result from either an increase in NK cell number or an activation by IFN of individual NK cells to a higher lytic capacity. Although we cannot distinguish between these mechanisms, it is clear that blast-NK cells do not contribute substantially to the activity.

In our short term in vivo tumor cell rejection assays, both YAC-1 cells and L-929 cells were cleared more efficiently from the lungs of carrier mice as compared to normals. The rejection of YAC-1 cells from the lung is thought to be mediated by NK cells (19). Talmadge et al. (4) showed that increased melanoma tumor cell rejection from the lung correlates with smaller mean tumor diameters and a smaller number of metastases. Furthermore, these investigators showed that acute LCMV infection rendered mice more resistant to growth and metastasis of tumors. This resistance was markedly decreased when NK-deficient beige mice were used. Recent work in our laboratory (27) has shown that mice acutely infected with LCMV reject tumor cells more efficiently in a short term $^{125}$IUDR assay. It therefore seems likely that carriers having elevated levels of NK cell-mediated lysis presumably due to chronic IFN activation could be more resistant to tumor growth and metastasis. To our knowledge, this is the first body of evidence indicating that persistent infection may enhance resistance to tumor implants. It should be noted that although the relatively NK-insensitive L-929 cells were lysed poorly in vitro by normal or LCMV carrier NK cells, carriers rejected them more efficiently than normals in vivo. In vivo rejection of targets refractory to detectable in vitro lysis has been reported previously (4).
LCMV is a natural mouse pathogen and the carrier state is very common among mice in the wild (28). Why has the virus persisted among mouse populations for many years? It is interesting to speculate that increased resistance to tumors could give carrier mice a survival advantage. Also, it is well known that LCMV carriers are resistant to many types of viruses including polio (29), leukemia (30), Western equine encephalitis (31), Eastern equine encephalitis (32), MM virus (33), polyoma (34), and VSV (35). This observation is consistent with the presence of IFN which could provide carriers with an additional survival advantage. Further, recent work in our laboratory indicates that NK cells may mediate natural resistance to a variety of viruses, and this resistance may increase if the NK cells are activated (Bukowski & Welsh, unpublished).

In summary, the LCMV carrier mice give us some insight into the effect of persistent infection on NK cells, IFN production, and tumor resistance. The carrier could be used as an in vivo model to study the effects of chronic IFN treatment on tumor resistance.
Acknowledgement

We thank Ms. Dottie Walsh for preparation of the manuscript.
References


## Table 1. Cytotoxic Activity Against YAC-1 Cells Mediated by Leukocytes From Normal and LCMV-Carrier BALB/c Mice

<table>
<thead>
<tr>
<th>Exp.</th>
<th>Age</th>
<th>Target Ratio</th>
<th>Effector to % Specific Release</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Normal</td>
</tr>
<tr>
<td>1</td>
<td>5 wks.</td>
<td>100</td>
<td>2.6 ± 1.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>33</td>
<td>2.6 ± 2.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>11</td>
<td>1.7 ± 0.6</td>
</tr>
<tr>
<td>2</td>
<td>8 wks.</td>
<td>100</td>
<td>3.7 ± 1.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>33</td>
<td>2.3 ± 2.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>11</td>
<td>2.6 ± 0.6</td>
</tr>
<tr>
<td>3</td>
<td>12 wks.</td>
<td>100</td>
<td>4.7 ± 3.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>33</td>
<td>3.0 ± 1.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>11</td>
<td>1.3 ± 1.2</td>
</tr>
<tr>
<td>4</td>
<td>26 wks.</td>
<td>100</td>
<td>2.4 ± 1.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>33</td>
<td>0.8 ± 1.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>11</td>
<td>0.5 ± 0.5</td>
</tr>
<tr>
<td>B.</td>
<td>5 wks.</td>
<td>100</td>
<td>19. ± 2.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>33</td>
<td>9.3 ± 0.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>11</td>
<td>5.0 ± 1.0</td>
</tr>
<tr>
<td>2</td>
<td>22 wks.</td>
<td>100</td>
<td>12. ± 3.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>33</td>
<td>9.0 ± 6.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>11</td>
<td>3.0 ± 2.0</td>
</tr>
</tbody>
</table>

*Cytotoxic activity of BALB/c spleen cells against YAC-1 targets in a standard 51Cr release assay. Each number represents the mean activity of at least 3 separate animals ± 1 S.D. A. The assay length was 6 hr and the spontaneous release was between 8 and 14%. B. The assay length was 16 hr, and the spontaneous release was between 16 and 24%.*
Table 2. Effect of Acute and Persistent Infection on Spleen Cell Cytotoxicity Against NK-Resistant L-929 Cells

<table>
<thead>
<tr>
<th>Type of Infection</th>
<th>% Specific $^{51}$Cr Release</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>YAC-1: 21.</td>
</tr>
<tr>
<td></td>
<td>L-929: 5.3</td>
</tr>
<tr>
<td>Persistent (LCMV carrier)</td>
<td>YAC-1: 63.</td>
</tr>
<tr>
<td></td>
<td>L-929: 4.7</td>
</tr>
<tr>
<td>Acute LCMV</td>
<td>YAC-1: 88.</td>
</tr>
<tr>
<td></td>
<td>L-929: 22.</td>
</tr>
</tbody>
</table>

*Splenic effector cells from an 8 wk. old BALB/c mouse, an age-matched LCMV carrier, or a mouse acutely infected for 3 days were mixed with targets at 100:1 in a 16 hr $^{51}$Cr release assay. Spontaneous release was between 20 and 27% for YAC-1 cells and 24-34% for L-929.*
Table 3. Characteristics of Cytotoxic Spleen Cells in LCMV-Carrier Mice<sup>a</sup>

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Treatment</th>
<th>% Specific 51Cr Release</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>None</td>
<td>20. ± 3.5</td>
</tr>
<tr>
<td></td>
<td>C'</td>
<td>15. ± 1.0</td>
</tr>
<tr>
<td></td>
<td>Anti-AGM&lt;sub&gt;1&lt;/sub&gt; + C'</td>
<td>-2.3 ± 2.1</td>
</tr>
<tr>
<td>2</td>
<td>None</td>
<td>65. ± 6.7</td>
</tr>
<tr>
<td></td>
<td>Anti-AGM&lt;sub&gt;1&lt;/sub&gt; in vivo</td>
<td>2.7 ± 3.1</td>
</tr>
<tr>
<td>3</td>
<td>None</td>
<td>36. ± 3.8</td>
</tr>
<tr>
<td></td>
<td>C'</td>
<td>34. ± 1.2</td>
</tr>
<tr>
<td></td>
<td>Anti-NK 1,2 + C'</td>
<td>13. ± 3.2</td>
</tr>
<tr>
<td>4</td>
<td>None</td>
<td>64. ± 2.3</td>
</tr>
<tr>
<td></td>
<td>C'</td>
<td>61. ± 1.2</td>
</tr>
<tr>
<td></td>
<td>Anti-thy-1,2 + C'</td>
<td>53. ± 2.9</td>
</tr>
</tbody>
</table>

<sup>a</sup>BALB/c carrier spleen leukocytes were mixed with YAC-1 cells in a standard 51Cr release assay at effector to target ratios of 100:1 (experiment 3 was 50:1). Assays were 16 hr long except for experiment 1, which was 6 hr long. The numbers represent the mean activities of 4 separate assay wells ± 1 S.D. In experiment 2, the mean activities of 3 mice are presented. Spontaneous release was 9-23%.
Table 4. Interferon Levels in LCMV-Carrier, Acutely Infected, And Normal Mice.

<table>
<thead>
<tr>
<th>Exp.</th>
<th>Mouse Strain</th>
<th>Infection</th>
<th>Interferon Titer log$_2$ (units/ml)$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>BALB/c</td>
<td>uninfected</td>
<td>&lt;2.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>carrier</td>
<td>5.7 ± 0.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>acutely infected</td>
<td>10. ± 0</td>
</tr>
<tr>
<td>2</td>
<td>SWR</td>
<td>uninfected</td>
<td>&lt;2.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>carrier</td>
<td>5.3 ± 0.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>acutely infected</td>
<td>12. ± 0</td>
</tr>
<tr>
<td>3</td>
<td>(BALB/c x SWR) F1</td>
<td>uninfected</td>
<td>&lt;2.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>carrier</td>
<td>5.3 ± 0.6</td>
</tr>
</tbody>
</table>

$^a$Each number represents the mean plasma IFN titer of 3 separate control or carrier mice or 2 acutely infected mice ± 1 S.D. The acutely infected mice received an intraperitoneal inoculation of $8 \times 10^4$ PFU of LCMV 3 days prior to sacrifice.
<table>
<thead>
<tr>
<th>Sample</th>
<th>Treatment</th>
<th>Interferon Titer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>log₂ (units/ml)</td>
</tr>
<tr>
<td>Type I IFN Standard</td>
<td>None</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>pH 2.0</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>Anti-type I IFN</td>
<td>&lt;2</td>
</tr>
<tr>
<td>Con A Supernatant (Type II IFN)</td>
<td>None</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>pH 2.0</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Anti-type I IFN</td>
<td>6</td>
</tr>
<tr>
<td>LCMV-Carrier Plasma</td>
<td>None</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>pH 2.0</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>Anti-type I IFN</td>
<td>&lt;2</td>
</tr>
</tbody>
</table>

*LCMV-carrier serum was pooled from five 5-month-old mice. The pH 2 treatment was carried out by overnight dialysis in pH 2.0 glycine HCl buffer, followed by an overnight dialysis in Hank's balanced salt solution. Anti-type I IFN treatment was carried out at room temperature for 30 min. Con A supernatant, a source of immune IFN, was drawn from a 48 hr culture containing 10⁶ splenic leukocytes/ml in RPMI assay medium supplemented with 2 ug/ml concanavalin A.*
Table 6: Effect of Poly I:C Treatment on NK Cell Activity in LCMV-Carrier and Normal Mice

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Effector: Target</th>
<th>BALB/c</th>
<th>SWR</th>
<th>(SWR X BALB/c) F1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Normal</td>
<td>Carrier</td>
<td>Normal</td>
</tr>
<tr>
<td>None</td>
<td>100</td>
<td>10.±3.5</td>
<td>35.±5.0</td>
<td>8.3±6.7</td>
</tr>
<tr>
<td></td>
<td>33</td>
<td>3.0±3.0</td>
<td>23.±5.6</td>
<td>2.0±1.0</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>1.3±2.3</td>
<td>15.±6.6</td>
<td>-0.7±0.6</td>
</tr>
<tr>
<td>Poly I:C</td>
<td>100</td>
<td>41.±4.2</td>
<td>48.±4.6</td>
<td>28.3±7.6</td>
</tr>
<tr>
<td></td>
<td>33</td>
<td>20.±3.8</td>
<td>32.±4.4</td>
<td>16.±5.5</td>
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<tr>
<td></td>
<td>11</td>
<td>11.±0.6</td>
<td>18.±3.5</td>
<td>7.0±4.0</td>
</tr>
</tbody>
</table>

*Cytotoxic activity of spleen cells against YAC-1 targets in a standard 6 hr ⁵¹Cr release assay. Some mice were injected intraperitoneally with 100 ug poly I:C 18-24 hr before the assay. Results are expressed as % specific ⁵¹Cr release. Each number represents the mean activity of 3 separate animals ± 1 S.D. Spontaneous release was between 10 and 16% of maximum. Male mice 6-8 weeks old were used.*
Table 7. Comparison of Plasma Interferon Levels Between Normal and LCMV-Carrier Mice Treated With Poly I:C

<table>
<thead>
<tr>
<th>Treatment</th>
<th>BALB/c Normal</th>
<th>BALB/c Carrier</th>
<th>SWR Normal</th>
<th>SWR Carrier</th>
<th>(SWR X BALB/c) F1 Normal</th>
<th>(SWR X BALB/c) F1 Carrier</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>&lt;2.0 ± .00</td>
<td>5.7 ± .66</td>
<td>&lt;2.0 ± .00</td>
<td>5.3 ± .22</td>
<td>&lt;2.0 ± .00</td>
<td>5.7 ± .22</td>
</tr>
<tr>
<td>Poly I:C</td>
<td>11. ± .66</td>
<td>8.7 ± .22</td>
<td>11. ± .22</td>
<td>8.3 ± .22</td>
<td>11. ± .22</td>
<td>8.7 ± .22</td>
</tr>
</tbody>
</table>

*In three separate experiments mice of the indicated strains were left untreated or injected with 100 µg poly I:C intraperitoneally 18-24 hr before plasma samples were taken. Interferon titers are expressed as $\log_2$ (units/ml) and each number is the mean titer of three separate animals ± 1 S.D. The age of the mice was between 6 and 8 weeks.*
Table 8. Clearance of Tumor Cells In Vivo: Comparison of Normal and Carrier Mice

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Mouse</th>
<th>Tumor Cell</th>
<th>% Counts Remaining in lung</th>
<th>% Specific $^{51}$Cr Release</th>
</tr>
</thead>
<tbody>
<tr>
<td>1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>BALB/c</td>
<td>YAC-1</td>
<td>39. ± 4.6</td>
<td>2.4 ± 1.5</td>
</tr>
<tr>
<td></td>
<td>normal</td>
<td>YAC-1</td>
<td>4.5 ± 2.6</td>
<td>30. ± 6.6</td>
</tr>
<tr>
<td>2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>BALB/c</td>
<td>L-929</td>
<td>71. ± 8.2</td>
<td>2.9</td>
</tr>
<tr>
<td></td>
<td>normal</td>
<td>L-929</td>
<td>44. ± 5.3</td>
<td>15.</td>
</tr>
</tbody>
</table>

<sup>a</sup>Exp. 1. Five normal male 6-month-old BALB/c mice and 5 age-matched carriers were injected i.v. with $10^6$ $^{125}$IUDR-labeled YAC-1 cells as described in materials and methods. Two hours later the mice were killed and their lungs were removed and counted in a gamma counter. The results are expressed as the % of total injected counts recovered from the lungs. The spleens from these mice were used as effectors in a 6 hr $^{51}$Cr release assay against YAC-1 cells. The results are expressed as % specific $^{51}$Cr release at a 100:1 effector to target ratio. Spontaneous release was 13%. Each number represents the mean activity of 5 separate mice ± 1 S.D.

<sup>b</sup>Six female 4 month old BALB/c mice and 6 age matched LCMV-carriers were injected with $5 \times 10^5$ $^{125}$IUDR-labeled L-929 cells. Lungs were harvested and counted 4 hr after injection, and spleen leukocytes were pooled from the individual mice for use in a cytotoxicity assay.
Legends to Figures

Figure 1. Size distribution of NK cells isolated from LCMV-carrier mice. Three groups of five BALB/c mice (3 months old) were sacrificed and their splenic leukocytes were separated into six fractions on the basis of size by elutriation. Each of these fractions (1 → 6 = smallest → largest) was used as an effector population against $^{51}$Cr labeled YAC-1 cells in a 14 hour assay at various effector to target ratios. The data obtained at an effector to target ratio of 11:1 are presented here. Spontaneous lysis was 16%. Acutely infected mice were injected i.p. with $8 \times 10^4$ pfu LCMV 3 days prior to sacrifice. At 100:1 effector to target ratio, unseparated normal spleen cells mediated 25% specific release, and acutely infected and carrier mice mediated 76% and 54%, respectively. Control mice (■—■), LCMV carrier mice (▲—▲), LCMV-acutely infected mice (●—●).
Figure 2. Size distribution of NK cells isolated from LCMV-carrier mice after activation with poly I:C in vivo. Three groups of three BALB/c mice (3 months old) were sacrificed and separated by elutriation as described in Fig. 1. Effector cell populations were tested in a 7 hr $^{51}$Cr release assay against YAC-1 cells. Results at an effector to target ratio of 12:1 are reported here. Spontaneous release was 8%. Spleen cells were isolated from untreated LCMV-carrier mice (▲—▲), LCMV-carrier mice injected i.p. with 100 ug poly I:C 24 hr prior to harvest (■—■), and control mice injected with 100 ug poly I:C 24 hr prior to harvest (○—○).
Figure 1
Figure 2
Natural Killer Cell Depletion Enhances Virus Synthesis and Virus-Induced Hepatitis In Vivo
Running Title:

Virus Infections in NK Cell-Depleted Mice
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Footnotes

1. This research was supported by USPHS research grant AI 17672, a
grant from the Council for Tobacco Research, Research Career
Development Award AI 00432 to R.W., and a Massachusetts State
Fellowship to J.B.

2. Abbreviations Used

   FBS - fetal bovine serum
   MEF - mouse embryo fibroblasts
   LCMV - lymphocytic choriomeningitis virus
   MCMV - murine cytomegalovirus
   MHV - mouse hepatitis virus
   BHK - baby hamster kidney
   NK - natural killer
   HBSS - Hank's balanced salt solution
   VSV - vesicular stomatitis virus
   TCID - tissue culture infective dose
   CPE - cytopathic effect
   PFU - plaque-forming units
   i.v. - intravenous
   i.p. - intraperitoneal
   IFN - interferon
   NRS - normal rabbit serum
   Asialo GM₁ - ganglio-n-tetraosylceramide
   Anti-AGM₁ - anti-asialo GM₁ antibody
   HSV - herpes simplex virus
   NC - natural cytotoxic
   EBV - Epstein - Barr virus
Abstract

The role of natural killer (NK) cells in the natural resistance of mice to infections by several viruses was examined. Mice were specifically depleted of NK cells by intravenous injection of rabbit antiserum to asialo GM\textsubscript{1}, a neutral glycosphingolipid present at high concentrations on the surface of NK cells. Control mice were left untreated or injected with normal rabbit serum. Four to six hours later, these mice were infected with lymphocytic choriomeningitis virus (LCMV), mouse hepatitis virus (MHV), mouse cytomegalovirus (MCMV), or vaccinia virus. The mice were sacrificed three days post-infection and assayed for virus in liver and spleen, spleen NK cell activity, and plasma interferon (IFN). All mice treated with anti-asialo GM\textsubscript{1} antibody had drastically reduced NK cell-mediated lysis. Correlating with NK cell depletion, these mice had significantly higher (up to 500-fold) titers of MCMV, MHV, or vaccinia virus in their livers and spleens as compared to control mice. NK cell-depleted MCMV and MHV-infected mice had higher levels of plasma IFN than controls, correlating with the higher virus titers. These NK cell-depleted, virus-infected mice had more extensive hepatitis as assayed by the number of inflammatory foci in their livers as compared to control virus-infected mice; these foci were also larger and contained more degenerating liver cells than those in control mice.

In contrast to the results obtained with MHV, MCMV, and vaccinia virus, NK cell depletion had no effect on virus titers in the early stages of acute LCMV infection or during persistent LCMV infection.
Mice depleted of NK cells had similar amounts of LCMV in their spleens and similar plasma IFN levels. Since this antibody to asialo GM₁ does not impair other detectable immunological mechanisms, these data support the hypothesis that NK cells act as a natural resistance mechanism to a number of virus infections but suggest that their relative importance may vary from virus to virus.
Introduction

While much is known about specific T cell-mediated and antibody-dependent responses to virus infections, less information is available concerning host factors contributing to natural immunity. Certain individuals are more susceptible to virus infections than others, and this can often be traced to either the genetic or physiological status of the individual. Of the various components of the natural immune system, i.e., natural antibody, complement, macrophages, polymorphonuclear leukocytes, and natural killer (NK) cells, the NK cells appear to be most sensitive to genotype, age, physiological, and hormonal variations (1). NK cell activity is augmented by interferon (IFN) (1) and reduced by corticosteroids (2), agents known to increase and decrease resistance to virus infections, respectively. The discovery that NK cells become activated during the early stages of virus infections in mice (3,4,5,6) led to the speculation that they may provide a first line of defense against dissemination of viruses (7). This hypothesis was reinforced by work showing that cultures of virus-infected cells are more sensitive than uninfected cultures to lysis by NK cells (8-13). This enhanced lysis of virus-infected cells may involve any of several mechanisms, including in situ activation of NK cells (9,10,14,15), enhanced binding of NK cells to viral glycoproteins displayed on the infected cell (12,16), and possibly to synergism between a cytopathic virus and a cytotoxic NK cell (17).

Direct evidence linking NK cells to resistance to virus infections has been lacking, though a number of indirect approaches have supported
the concept. NK cell development is dependent on the bone marrow, and resistance to Friend leukemia virus (18), herpes simplex 1 virus (HSV), (14) and mouse hepatitis virus (MHV) (20,21) has been shown to depend on a bone marrow cell having features similar to NK cells, including age of maturation, independence of the thymus, and sensitivities to various drugs. With certain viruses the susceptibility to infection varies markedly with the strain of mouse, and with mouse cytomegalovirus (MCMV) infections the resistance correlates with high NK cell activity (22,23). Virus-infected tumor cells are rejected from athymic nude mice more efficiently than are uninfected tumor cells in a manner correlating with in vitro sensitivity of those tumor cells to NK cell-mediated lysis (16). Mice are more highly susceptible to virus infections when NK cell activity is low, such as at birth or under various types of immunosuppression (24). While suggestive, none of the above studies convincingly implicates NK cells as effectors of natural resistance to virus infections, as other components of the immune response were not ruled out. Because methods for specifically depleting NK cell activity in vivo have not been available, it has not been possible to state with any degree of certainty that NK cells were responsible for resistance.

A recently prepared reagent, antibody to asialo GM₁ (25), appears to selectively deplete NK cell activity in vivo (26,27). Asialo GM₁ is a neutral glycosphingolipid present at high quantities on the surface of NK cells (25,26,28). This antiserum in the presence of complement reduces virtually all spleen NK cell activity and eliminates
virtually all the large granular lymphocytes, while lysing less than 5% of the total number of spleen leukocytes (25,28). The fluorescein-ated antibody does not stain granulocytes, though it does react with a subpopulation of monocytes (26). When administered in vivo it markedly reduces NK cell activity while having no effect on cytotoxic macrophage (27), cytotoxic T cell (27), or natural cytotoxic (NC) cell (unpublished data) functions. Further, the concanavalin A response (26) and the percentage of Thy-1-positive (26) and surface immunoglobulin-positive (unpublished data) cells found in the spleen of in vivo-treated mice are unaffected (26). Habu et. al. have shown that mice treated with this reagent become more sensitive to the outgrowth of NK-sensitive tumor implants (26).

We report here a survey of the effects of this reagent in mice on virus synthesis and hepatic pathology induced by viruses representing four distinct groups. These include lymphocytic choriomeningitis virus (LCMV), arenavirus group, MCMV, herpesvirus group, MHV, coronavirus group, and vaccinia virus, poxvirus group. The results show that this selective NK cell depletion markedly enhances the infection of three of the four viruses tested and provide the most convincing evidence thusfar indicating that NK cells may play a major role in the natural immunity to certain viruses.
Materials and Methods

Animals. C3H/St mice were purchased from West Seneca Laboratories, West Seneca, N.Y. C57BL/6 (bg/+ and bg/bg) and BALB/c byJ mice were originally purchased from Jackson Laboratories, Bar Harbor, ME, and then bred in our own facilities. Male mice 6-12 weeks old were used in these experiments unless otherwise indicated.

Cells. YAC-1 cells were derived from a Moloney leukemia virus-induced lymphoma in A/Sn mice and maintained in RPMI-1640 medium (Gibco, Grand Island, NY) supplemented with antibiotics, glutamine and 10% heat-inactivated fetal bovine serum (FBS) (M.A. Bioproducts, Walkerville, MD). WEHI-164 cells, a target for NC cells (29), were obtained from Dr. R. Burton and propagated in RPMI medium supplemented as above. Vero cells are a continuous monkey kidney cell line, and L-929 cells are a continuous liver cell line from C3H mice. Mouse embryo fibroblasts (MEF) were obtained as previously described (30) from C57BL/6 mouse embryos. These cells were maintained in minimal essential medium (MEM) (Gibco) with the same additives as listed above. Mouse BALB-3T3 and baby hamster kidney (BHK) 21/13S cells were maintained in Dulbecco's modified essential medium (Gibco) supplemented with 10% tryptose phosphate broth and the same additives as listed above.

Viruses. The lymphocytic choriomeningitis virus (LCMV) used in these studies was the Armstrong strain, which was grown in BHK cells (31). Vaccinia virus, WR strain, was obtained from Dr. Robert Singer, U. Mass. Medical School. This virus was grown in mouse L-929 cells,
yielding titers of about $10^9$ pfu/ml. MHV (strain A-59) was obtained from Dr. K.V. Holmes, USUHS, Bethesda, MD (32). The virus was maintained by passage in 3T3 cells. Before use in experiments, tissue culture - grown MHV was passed once in vivo by intracerebral inoculation of C3H/St or C57BL/6 mice with $10^5$ pfu. Three days later, the livers were homogenized in a 10% suspension and cleared by centrifugation. A stock containing about $5 \times 10^5$ pfu/ml was routinely obtained. The Smith strain of MCMV was obtained from Dr. John Nedrud, University of North Carolina, Chapel Hill, N.C. (33). This virus was maintained by in vivo passage in weanling BALB/c mice. Salivary glands from mice inoculated 2-3 weeks previously with $10^4$ pfu MCMV were homogenized in a 10% suspension and cleared by centrifugation. This procedure yielded stocks with titers of $5 \times 10^6 - 2 \times 10^7$ pfu/ml.

Establishment of Mice Persistently Infected with LCMV. BALB/c mice were injected intraperitoneally with $2.4 \times 10^4$ PFU of LCMV within 24 hours of birth (34). These mice became persistently infected for life and gave birth to congenitally persistently infected offspring. In these experiments, those congenitally infected mice were used.

Histopathology. Liver sections were fixed in formalin, embedded in glycol methacrylate, cut at 2.5-6 μ and stained with acid fuchsin - toluidine blue - eosin. Inflammatory foci/10 low power fields were determined by observing the sections with an American Optical one-ten microscope equipped with a 10x objective and 10x wide field oculars, yielding a field diameter of 1.7 mm.
Anti-asialo GM\textsubscript{1} Antiserum. Rabbit antiserum to bovine asialo GM\textsubscript{1} was prepared as previously described (25). Briefly, purified asialo GM\textsubscript{1} was emulsified with complete Freund's adjuvant and injected into each footpad of a rabbit. The rabbit was given a booster shot four weeks later and then bled two weeks later. This antiserum has previously been shown to selectively deplete NK cells \textit{in vivo} (26,27) and \textit{in vitro} (25,28). To deplete NK cells \textit{in vivo}, anti-asialo GM\textsubscript{1} was diluted 1:10 in RPMI medium and given intravenously in a volume of 0.2 ml.

Interferon Assay. Blood was collected in heparinized Natelson tubes from the retro-orbital sinus of mice anaesthetized with ether. Plasma was obtained by centrifugation, and some samples were dialyzed overnight in pH 2 glycine-HCl buffer followed by an overnight dialysis in Hank's balanced salt solution (HBSS) (Gibco). Samples were titrated by 2-fold serial dilutions in a 96 well flat bottom microtiter plate to which L-929 cells were added at 3 x 10\textsuperscript{4} cells/well. Eighteen to twenty-four hours later, the wells were challenged with 100 TCID\textsubscript{50} units of vesicular stomatitis virus (VSV). IFN titers were expressed as the log\textsubscript{2} of the highest reciprocal dilution resulting in 50% reduction in cytopathic effect (CPE). Results are expressed as the geometric mean titers of four separate animals titrated for IFN individually.

Cytotoxicity Assay. Assay medium was RPMI medium supplemented with 0.1 M N-2-hydroxyethyl piperazine - N\textsuperscript{'}- 2 - ethane sulfonic acid (HEPES) (Sigma Chemical Co., St. Louis, MO), 10% FBS, glutamine, and
antibiotics. The assay was performed as described (35). Briefly, target cells labeled with 100 uCi sodium chromate ($^{51}\text{Cr}$; Amersham Corp., Arlington Hts., IL) for 1 hr at 37°C were washed and mixed with $10^6$ effector cells in round-bottom microtiter wells at $10^4$ target cells/well. For spontaneous release determination, medium was added to the wells, and 1% Nonidet P-40 was added for maximum release determination. Plates were incubated for 6 to 16 hours at 37°C in a humidified atmosphere of 5% CO$_2$, 95% air. At the end of the incubation, plates were centrifuged at 200 g for 5 min, and 0.1 ml of the supernatant was collected and counted for radioactivity in a Beckman Gamma 5500 counter (Beckman Instruments, Palo Alto, CA). Data are expressed as % specific release:

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

Spontaneous release was between 8 and 17%. Standard deviations of quadruplicate replica samples were < 10% of the mean and were not listed in the tables. Results of cytotoxicity assays using several individual mice are tabulated as the mean % specific $^{51}\text{Cr}$ release of four separate animals ± one standard deviation.

**Virus Titration.** Spleen and liver virus titers were determined by using a 10% homogenate of tissue taken from individual mice. The number of plaque forming units (pfu) per spleen or per gram liver was determined by plaque assays using Vero cells for LCMV and vaccinia virus, MEF for MCMV, and L-929 cells for MHV. Results are reported as
the geometric mean titers, i.e. the arithmetic averages of the logs of four separate animals titrated for virus individually. P values represent the significance of the differences of the means between the designated sample and the normal non-antibody-treated control, and were calculated using Student's t-test.

Experimental Procedure. The protocol for the experimental design is shown in Figure 1. Mice in groups of 4 were injected i.v. with 0.2 ml anti-asialo GM1 diluted 1:10. In some experiments, an additional group of mice was injected in the same manner with normal rabbit serum (NRS). Four to six hours later, these mice and 4 age-and sex-matched controls were injected i.p. with LCMV or MCMV, or i.v. with vaccinia virus. MHV was given either i.p. or i.v. Three days postinfection, the mice were sacrificed and were assayed individually for spleen NK cell-mediated cytotoxicity, PFU in the spleen and liver, plasma IFN, and hepatic inflammation. For each virus used in this study, this experimental procedure was performed on 3-6 separate occasions, and the results of representative experiments are presented.
Results

Activation of NK Cells During Acute Viral Infections. To examine NK cell activation under conditions employed for NK cell depletion studies, mice were injected with MHV, vaccinia virus, MCMV, or LCMV and sacrificed three days later. Spleen cell suspensions were prepared and cytotoxicity assays were performed using YAC-1 cells as targets. Spleen leukocytes from mice infected with any of the four viruses lysed target cells more efficiently than control spleen cells (Table I). This confirms previous observations (3, 4, 36, 37). Similarly, spleen cells from mice persistently infected with LCMV displayed a higher degree of lysis than control spleen cells (Table I), confirming our recent observation (34). At the beginning of the present studies we tested the efficacy of the antiserum to asialo GM₁ on the mice used and found that it inhibited endogenous and virus-induced NK cell activity (Tables II-VII), had no effect on the formation of LCMV-specific cytotoxic T cells, and had no effect on the lysis of WEHI-164 cells, a target for the NC cell (29) (data not shown).

Effect of NK Cell Deletion on LCMV Infection. Treatment with anti-asialo GM₁ markedly depleted detectable LCMV-induced NK cell activity in C3H/St and C57BL/6 mice, but this treatment had no significant effect on spleen LCMV titers or plasma IFN levels 3 days postinfection (Table II). Under these conditions of infection no LCMV was found in the liver, and the negative liver data are not tabulated. BALB/c mice persistently infected with LCMV also did not have significant differences
in spleen virus titers when compared to NK cell-depleted mice (Table III). When these persistently infected mice were bled from the retroorbital sinus on successive days, LCMV titers in the plasma increased in both the normal and NK cell-depleted mice over this three day period. This is a consistent finding in both treated and untreated mice, and the reason for it is unknown. However, NK cell depletion had no effect on this observation or on the viral titers. (Table IV).

Effect of NK Cell Depletion on MCMV Infection. In contrast to the results obtained with acute LCMV infection, NK cell depletion with anti-asialo GM₁ resulted in enhanced MCMV synthesis in the spleen and the liver as compared to untreated controls. Table V shows that C57BL/6 mice treated with anti-asialo GM₁ synthesized 30-500 fold more MCMV in their spleens, and 10-30 fold more MCMV in their livers as compared to control mice. Spleen and liver MCMV titers in NK cell-deficient bg/bg mice were also elevated (Table V), confirming the work of Shellam et. al. (5). When C3H/St mice were challenged with the same virus dose (5 x 10³ PFU) given to C57BL/6 mice, NK cell depletion had no significant effect on virus titers in the C3H/St mice. However, when the MCMV challenge dose was increased 5-fold, NK cell-depleted C3H/St mice had significantly higher virus titers in their spleens and livers as compared to controls (Table V). Intravenous injection of normal rabbit serum did not affect either NK cell-mediated lysis or virus titers in MCMV-infected C57BL/6 mice (data not shown).
High plasma IFN titers correlated with high MCMV titers and not with the presence of NK cells. Table V shows that C57BL/6 mice infected with MCMV and depleted of NK cells with antibody had slightly higher IFN levels than control MCMV-infected mice, correlating with higher virus titers. This indicates that the antibody to asialo $\text{GM}_1$ does not enhance virus synthesis by inhibiting IFN production.

**Effect of NK Cell Depletion on MHV Infection.** Results similar to those obtained with MCMV are shown for MHV in Table VI. Anti-asialo $\text{GM}_1$-treated mice had 4-50 fold higher spleen MHV titers and 4-500 fold higher liver MHV titers as compared to controls. As seen with MCMV, NK cell depletion had a greater effect on virus titers in C57BL/6 mice than it did in C3H/St mice. In both strains, NK cell depletion resulted in higher plasma IFN titers, correlating with high MHV titers. Infection with MHV by day 3 resulted in the formation of 5-10 white necrotic foci on the surface of the liver. NK cell-depleted mice had 10-20 fold more of these foci on their livers as compared to controls (data not shown), correlating with the higher virus titers seen in these mice.

**Effect of NK Cell Depletion on Vaccinia Virus Infection.** Table VII shows that NK cell depletion had a significant enhancing effect on vaccinia virus synthesis in the livers and spleens of C57BL/6 mice and in the spleens but not the livers of C3H/St mice. At this point in the infection, plasma IFN was undetectable, correlating with low virus titers under these conditions of infection.
Histopathology. Liver sections were examined for pathology in mice infected with the viruses whose synthesis had been altered by antibody to asialo GM\_1 or mice treated with antibody alone. Treatment with antibody to asialo GM\_1 alone did not result in any liver pathology in uninfected C57BL/6 mice. In control MCMV-infected mice, inflammatory foci were either arranged around degenerating hepatocytes or else degenerating hepatocytes were at the periphery of the foci. The lesions ranged from 1-6 hepatic cords in width (Figs. 2a, 2c). In the smaller lesions, mononuclear cells, presumably Kupffer cells, were predominant (Fig. 2e). The larger lesions contained more degenerating hepatocytes and neutrophils (Fig. 2f). In MCMV-infected anti-asialo GM\_1-treated mice inflammatory foci were more numerous (Table VIII, Figs. 2b, 2d) and larger. Intranuclear eosinophilic viral inclusion bodies were frequently seen (Fig. 2c). Large, confluent necrotic areas infiltrated by neutrophils were also present in the MCMV-infected mice treated with anti-asialo GM\_1 (Fig. 2b).

In control mice infected with MHV the inflammatory lesions ranged from small (2-3 cords wide) to large lesions, 5-8 hepatic cords in width (Fig. 3a). The large lesions contained many degenerating acidophilic hepatocytes, nuclear debris, neutrophils and mononuclear cells. The smaller lesions contained fewer neutrophils, relatively more mononuclear cells and fewer degenerating hepatocytes. In MHV-infected mice treated with anti-asialo GM\_1, more inflammatory foci were seen than in control mice (Table VIII). These foci tended to be larger, contained more degenerating hepatocytes, and in some areas the in-
flammation was almost confluent (Fig. 3b). Some of the livers from NK cell-depleted MHV-infected mice had areas of confluent hepatic necrosis and inflammation.

Livers from control mice infected with vaccinia virus had small inflammatory foci consisting of 1 or 2 degenerating hepatocytes surrounded by mononuclear cells (Fig. 4a). In animals infected with vaccinia virus and treated with anti-asialo GM1, the inflammatory foci were more numerous (Table VIII) and larger, with a width of 2-3 hepatic cords (Fig. 4b). These lesions contained more degenerating hepatocytes, neutrophils and nuclear debris.
Discussion

The results indicate that depletion of NK cells by antibody to asialo GM₁ renders mice more susceptible to 3 out of 4 viruses examined. The virus titers were up to 500-fold higher in the NK cell-depleted mice, and pathological lesions in the livers, i.e., the degree of hepatitis, correlated with virus production. Because this antibody appears to selectively deplete mice of NK cells in vivo, (26) we favor the hypothesis that the effects produced by this antibody are a result of NK cell depletion. We cannot rule out that another untested effector mechanism is not influenced by this treatment, but most systems (cytotoxic macrophages, polymorphonuclear leukocytes, NC cells, cytotoxic T cells, surface immunoglobulin-bearing cells, antigen presenting cells, etc.) appear normal. This is thus the best evidence to date that NK cells have antiviral properties in vivo and supports previous results arguing for or against a role for NK cells in murine infections with LCMV, MCMV, and MHV.

In the present study the synthesis of LCMV in either acutely or persistently infected mice was not influenced by the NK cell depletion. We have never found any indication that NK cells contribute to resistance to LCMV. Most strain differences in regards to susceptibility to LCMV are minor and do not correlate with NK cell activity (38,39). NK cell-deficient mice synthesize amounts of LCMV comparable to normal heterozygous littermates at early stages of the infection (40). LCMV-infected cells are not particularly sensitive to NK cell-mediated
lysis in vitro (12), and while they are lysed preferentially when injected in vivo, the effector cell responsible for this lysis (though undefined) is not an NK cell (41).

Antibody to asialo GM₁ rendered mice more sensitive to MCMV, and this correlates with previous investigations. Strains of mice with high NK cell activity are more resistant to MCMV infection than strains with low NK cell activity (22). NK cell-deficient beige mice are highly susceptible to MCMV, and resistance to MCMV can be transferred by bone marrow from mice with normal NK cell levels (23). Hydrocortisone treatment renders mice more susceptible to MCMV in a manner which correlates with reduced NK cell activity caused by the drug (42). MCMV is a member of the herpesvirus group, and Lopez and coworkers have shown correlations in mouse and man between NK cell levels and resistance to HSV-1 infection (19). In the mouse model resistance to HSV is dependent on the bone marrow, which is essential for NK cell development (19). Recently, Habu et. al. have shown that mice depleted of NK cells with antibody to asialo GM₁ become more susceptible to HSV-1 infection³. In vitro studies have suggested that outgrowth of B cells infected with Epstein-Barr (EBV) virus, another member of the herpesvirus group, may be influenced by NK cells (43) and that these NK cells may preferentially lyse EBV-infected targets (11). These data collectively support the hypothesis that NK cells may play major roles in resistance to various members of the herpesvirus group.

MHV was another virus whose infection was enhanced by antibody to asialo GM₁. This is consistent with previous work showing that resistance
to MHV$_3$ (a different but related strain) requires a bone marrow cell which matured at the third week of life (21). Newborn mice, which were sensitive to MHV$_3$, could be protected by cell transfers which included a T cell, macrophage, and bone marrow (NK?) cell component (21).

Though NK cells are activated to high levels in vaccinia virus-infected mice (31), there are little data either supporting or refuting the concept that the NK cells play a role in resistance. Our data indicate that NK cells do provide resistance to vaccinia virus and may even be the difference between a successful or abortive infection. In some of our experiments vaccinia virus pfu's were only detected in mice treated with antibody to asialo GM$_1$.

NK cells could provide resistance to virus infections by preferentially lysing virus-infected cells in vivo, thereby inhibiting the spread of virus. It has also been suggested that they could have antiviral properties by secreting large amounts of IFN (44). Our data, however, indicate that IFN production is not inhibited when NK cells are depleted in vivo. In contrast, there was usually more IFN produced by NK cell-depleted animals, probably correlating with the greater virus stimulus. Though not extensively studied it appeared from our work that depletion of NK cells had more of an effect on virus titers in C57BL/6 mice than in C3H/St mice. The reason for this is not known. One possible explanation is that C3H mice are reported to have higher levels of NK cells than C57BL/6 mice (45). Perhaps the
antibody was not totally effective in eliminating the higher levels of tissue-associated NK cells in the high NK strain (C3H) mice.

Why the synthesis of MCMV, MHV and vaccinia virus is enhanced by NK cell depletion while synthesis of LCMV is not is unknown at present. Since LCMV causes a persistent infection in mice in nature, LCMV may have evolved in such a way as to escape NK cell surveillance. To establish an infection in mice for our studies, MCMV and MHV had to first be adapted by in vivo passage, and vaccinia virus, even at high dose inoculum, replicated poorly in vivo. This suggests that these viruses had to overcome barriers that did not inhibit LCMV. There may, however, be other reasons why LCMV would be different from the other viruses. LCMV is a relatively noncytopathic virus, while MCMV, MHV, and vaccinia virus are highly cytopathic. NK cells may preferentially lyse target cells which have been stressed by a cytopathic virus. A related issue is that of IFN protection. IFN not onlyactivates NK cells but also protects target cells from lysis mediated by activated NK cells (41). Thymocytes and tumor cells taken from LCMV-infected mice are resistant to NK cell-mediated lysis (47,48). IFN-mediated protection requires cellular RNA and protein synthesis (47). Hence, cells infected with cytopathic viruses may not become IFN-protected because cellular macromolecular synthesis is inhibited (49), while cells infected with noncytopathic viruses may become IFN-protected. IFN will protect LCMV-infected L-929 cells from activated NK cell-mediated lysis in vitro (Welsh, unpublished data).
It is thus becoming clear that NK cells may represent a potent effector arm of the host response. The evidence that they provide resistance to implanted syngeneic tumors is overwhelming (1). They may also be a major mediator of antibody-dependent cell-mediated cytotoxicity, which may play an important role in immunity to infection after antibody is synthesized (1). Our results here and those of Habu et. al. 3, while not totally conclusive, provide the strongest evidence to date that NK cells are mediators of natural resistance to virus infections. However, in the unlikely event that another undefined mechanism is responsible for our results, it remains interesting that a potent antiviral natural resistance mechanism is eliminated by this antibody to asialo GM1.
Acknowledgements

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References


Table I. Augmentation of NK Cell Activity During Acute and Persistent Viral Infections

<table>
<thead>
<tr>
<th>Exp.</th>
<th>Infection</th>
<th>% Specific $^{51}$Cr Release From YAC-1 Targets</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. a</td>
<td>Acute MCMV None</td>
<td>71.</td>
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<tr>
<td></td>
<td>None</td>
<td>40.</td>
</tr>
<tr>
<td>2. a</td>
<td>Acute MHV None</td>
<td>48.</td>
</tr>
<tr>
<td></td>
<td>None</td>
<td>30.</td>
</tr>
<tr>
<td>3. a</td>
<td>Acute Vaccinia Virus None</td>
<td>58.</td>
</tr>
<tr>
<td></td>
<td>None</td>
<td>41.</td>
</tr>
<tr>
<td>4. a</td>
<td>Acute LCMV None</td>
<td>63.</td>
</tr>
<tr>
<td></td>
<td>None</td>
<td>27.</td>
</tr>
<tr>
<td>5. b</td>
<td>Persistent LCMV None</td>
<td>35.</td>
</tr>
<tr>
<td></td>
<td>None</td>
<td>10.</td>
</tr>
</tbody>
</table>

a. C57BL/6 mice were injected 3 days previously with virus as stated in Materials and Methods, and spleen cells were used at a 100:1 effector to target ratio.

b. Three-month-old congenitally infected BALB/c mice and age-matched controls were used.
Table II. Lack of Effect of NK Cell Depletion on Virus and IFN Titers During Acute LCMV Infection

<table>
<thead>
<tr>
<th>Exp.</th>
<th>Mouse</th>
<th>anti-AGM</th>
<th>%NK Lysis</th>
<th>log₁₀ pfu/ Spleen</th>
<th>log₁₀ pfu/ g.Liver</th>
<th>log₂ IFN U/ ml. Plasma</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>C57BL/6</td>
<td>-</td>
<td>63. ± 4.7</td>
<td>5.7 ± 0.2</td>
<td>&lt;2.0</td>
<td>11. ± 0.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+</td>
<td>0.5 ± 0.3</td>
<td>5.6 ± 0.2</td>
<td>&lt;2.0</td>
<td>12. ± 0.6</td>
</tr>
<tr>
<td>2.</td>
<td>C3H/St</td>
<td>-</td>
<td>49. ± 3.4</td>
<td>6.0 ± 0.2</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+</td>
<td>7.0 ± 4.1</td>
<td>5.7 ± 0.5</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

a Mice were inoculated with 8 x 10⁴ pfu LCMV i.p.

b N.D., not done.
Table III. Lack of Effect of NK Cell Depletion On Spleen Virus Titers in BALB/c Mice Persistently Infected with LCMV\textsuperscript{a}

<table>
<thead>
<tr>
<th>Anti-AGM\textsubscript{1}</th>
<th>% NK Lysis</th>
<th>(\log_{10}\text{Pfu/Spleen})</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>65. ± 6.7</td>
<td>6.7 ± 0.2</td>
</tr>
<tr>
<td>+</td>
<td>2.7 ± 3.1</td>
<td>6.8 ± 0.1</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Groups of four 5-month-old BALB/c mice persistently infected with LCMV were injected i.v. with anti-asialo GM\textsubscript{1} or left untreated. Three days later, mice were sacrificed and NK cell-mediated cytotoxicity and spleen virus titers were determined (see Materials and Methods).
Table IV. Lack of Effect of NK Cell Depletion on Blood Virus Titers in BALB/c Mice Persistently Infected With LCMV.

<table>
<thead>
<tr>
<th>Day Post-Treatment</th>
<th>No Treatment</th>
<th>Anti-Asialo GM₁-Treated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.95</td>
<td>5.08</td>
</tr>
<tr>
<td>1</td>
<td>5.25</td>
<td>5.00</td>
</tr>
<tr>
<td>2</td>
<td>5.45</td>
<td>5.71</td>
</tr>
<tr>
<td>3</td>
<td>5.83</td>
<td>5.95</td>
</tr>
</tbody>
</table>

a. Blood samples from mice were taken just before treatment with anti-asialo GM₁ and for three days thereafter. Each column represents successive bleeds from an individual mouse.
Table V. Enhancement of Virus Synthesis and IFN Levels During Acute MCMV Infection in NK Cell-Depleted Mice.

<table>
<thead>
<tr>
<th>Exp.</th>
<th>Mouse</th>
<th>Anti-AGM</th>
<th>% NK Lysis</th>
<th>log_{10} pfu/ Spleen</th>
<th>log_{10} pfu/ g. Liver</th>
<th>log_2 IFN U/ml. Plasma</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>C57BL/6(bg/+  )</td>
<td>-</td>
<td>51. ± 14.</td>
<td>1.4 ± 0.2</td>
<td>4.2 ± 0.2</td>
<td>8.5 ± 0.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+</td>
<td>3.8 ± 2.6</td>
<td>4.1 ± 0.1(^b)</td>
<td>5.5 ± 0.3(^b)</td>
<td>10. ± 1.0(^d)</td>
</tr>
<tr>
<td></td>
<td>C57BL/6(bg/bg)</td>
<td>-</td>
<td>9.5 ± 5.3</td>
<td>3.9 ± 0.3(^b)</td>
<td>5.8 ± 0.2(^b)</td>
<td>11. ± 0.5(^b)</td>
</tr>
<tr>
<td>2(^e)</td>
<td>C57BL/6 (bg/+ )</td>
<td>-</td>
<td>46. ± 19.</td>
<td>3.2 ± 0.2</td>
<td>4.9 ± 0.2</td>
<td>7.7 ± 0.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+</td>
<td>1.5 ± 1.5</td>
<td>4.5 ± 0.1(^b)</td>
<td>6.0 ± 0.3(^b)</td>
<td>11. ± 0.6(^b)</td>
</tr>
<tr>
<td>3</td>
<td>C3H/St</td>
<td>-</td>
<td>71. ± 6.1</td>
<td>3.6 ± 0.2</td>
<td>N.D.(^f)</td>
<td>11. ± 0.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+</td>
<td>7.6 ± 2.9</td>
<td>3.4 ± 0.1</td>
<td>N.D.</td>
<td>10. ± 0.6</td>
</tr>
<tr>
<td>4(^a)</td>
<td>C3H/St</td>
<td>-</td>
<td>64. ± 6.8</td>
<td>3.7 ± 0.2</td>
<td>4.6 ± 0.1</td>
<td>8.7 ± 0.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+</td>
<td>0.8 ± 0.3</td>
<td>4.6 ± 0.1(^b)</td>
<td>5.0 ± 0.1(^c)</td>
<td>8.5 ± 0.6</td>
</tr>
</tbody>
</table>

a. In experiment 4, mice were challenged i.p. with 2.5x10\(^4\) pfu MCMV. In all other experiments, the dose was 5x10\(^3\) pfu.
b. P<.001
c. P<.01
d. P<.05
e. Female mice were used in this experiment.
f. N.D., not done.
Table VI. Enhancement of Virus Synthesis and IFN Levels During Acute MHV Infection in NK Cell-Depleted Mice

<table>
<thead>
<tr>
<th>Exp.</th>
<th>Mouse</th>
<th>Anti-AGM</th>
<th>% NK Lysis</th>
<th>log(_{10}) pfu/ Spleen</th>
<th>log(_{10}) pfu/ g. Liver</th>
<th>log(_{2}) IFN U/ ml. Plasma</th>
</tr>
</thead>
<tbody>
<tr>
<td>1(^a)</td>
<td>C57BL/6</td>
<td>-</td>
<td>48. ± 7.5</td>
<td>1.7 ± 0.9</td>
<td>3.7 ± 0.8</td>
<td>5.0 ± 2.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+</td>
<td>16. ± 9.7</td>
<td>3.4 ± 0.3(^c)</td>
<td>5.6 ± 0.2(^d)</td>
<td>9.5 ± 0.5(^c)</td>
</tr>
<tr>
<td>2(^b)</td>
<td>C3H/St</td>
<td>-</td>
<td>60. ±12.</td>
<td>2.6 ± 0.1</td>
<td>4.2 ± 0.3</td>
<td>7.0 ± 0.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+</td>
<td>2. ± 2.0</td>
<td>3.1 ± 0.1(^e)</td>
<td>4.7 ± 0.4</td>
<td>9.8 ± 0.5(^e)</td>
</tr>
</tbody>
</table>

a. Mice were infected with 5 x 10\(^4\) pfu MHV i.p.
b. Mice were infected with 1.5 x 10\(^5\) pfu MHV i.v.
c. P < .001
d. P < .01
e. P < .02
Table VII. Enhancement of Virus Synthesis During Acute Vaccinia Virus Infection in NK Cell-Depleted Mice

<table>
<thead>
<tr>
<th>Exp.</th>
<th>Mouse</th>
<th>Anti-AGM</th>
<th>% NK Lysis</th>
<th>(\log_{10}) pfu/1 g. Liver</th>
<th>(\log_{10}) pfu/1 g. Liver</th>
<th>(\log_{2}) IFN U/ml. Plasma</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>C57BL/6</td>
<td>-</td>
<td>58. + 8.2</td>
<td>&lt;2.0</td>
<td>&lt;2.0</td>
<td>&lt;2.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+</td>
<td>2.4 + 2.1</td>
<td>3.7 + 0.1(^b)</td>
<td>2.8 + 0.3(^c)</td>
<td>&lt;2.0</td>
</tr>
<tr>
<td>2</td>
<td>C3H/St</td>
<td>-</td>
<td>34. + 4.3</td>
<td>2.9 + 0.1</td>
<td>2.7 + 0.4</td>
<td>N.D.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+</td>
<td>7.8 + 4.7</td>
<td>3.3 + 0.3(^d)</td>
<td>3.3 + 0.6</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

a. Mice were infected with 5 x 10^6 pfu of vaccinia virus i.v.
b. \(P < .001\)
c. \(P < .02\)
d. \(P < .05\)
Table VIII. Increase in Inflammatory Foci in the Livers of NK Cell-Depleted Mice

<table>
<thead>
<tr>
<th>Virus Infection</th>
<th>Inflammatory Foci per 10 low power fields&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Control&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Anti-asi alo GM&lt;sub&gt;1&lt;/sub&gt;&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCMV</td>
<td>66</td>
<td>158</td>
<td>(13-115)&lt;sup&gt;c&lt;/sup&gt; (150-166)</td>
</tr>
<tr>
<td>MHV</td>
<td>5.0</td>
<td>49</td>
<td>(3-10) (10-109)</td>
</tr>
<tr>
<td>Vaccinia</td>
<td>1.2</td>
<td>20</td>
<td>(&lt;1-3) (3-23)</td>
</tr>
</tbody>
</table>

a. The numbers represent the mean number of inflammatory foci in 10 low power fields utilizing an American optical one-tenth microscope with a 10x objective and 10x wide field oculars, yielding a field diameter of 1.7 mm.


c. The numbers in parentheses represent the range of foci seen in individual liver sections.
Figure Legends

Figure 1. Protocol used in NK cell depletion studies.

Figure 2. Liver sections from MCMV-infected mice. A, control mouse liver showing 3 inflammatory foci (arrows) arranged around degenerating hepatocytes (Magnification = 100 X); B, anti-asialo GM$_1$-treated mouse liver showing a small inflammatory focus (arrow) and a large confluent area of hepatic necrosis (double arrows) (Magnification = 100 X); C, control mouse liver showing a small inflammatory focus (arrow) (Magnification = 160 X); D, anti-asialo GM$_1$-treated mouse liver showing multiple areas of inflammation arranged around degenerating hepatocytes. Cells with intranuclear inclusion are easily seen (arrows) (Magnification = 160 X); E, Control mouse liver showing a small inflammatory focus composed predominantly of mononuclear cells (Magnification = 400 X); F, a large lesion from an anti-asialo GM$_1$-treated mouse liver containing neutrophils, mononuclear cells and many degenerated necrotic hepatocytes (Magnification = 400 X).

Figure 3. Liver sections from MHV-infected mice. A, control mouse liver showing a single, well defined, large focus of inflammation (arrow); B, an anti-asialo GM$_1$-treated mouse liver showing a single large irregular lesion (double arrows) and portions of 2 other lesions (arrows). The inflammation is almost confluent (Magnification = 160 X).

Figure 4. Liver sections from vaccinia virus-infected mice. A, control mouse liver showing a single small lesion (arrow); B, an anti-asialo GM$_1$ treated mouse liver showing 2 medium-sized lesions (arrows) (Magnification = 160 X).
Anti-AGM

4-6 hours

N=4

Virus

3 days

LIVER, SPLEEN VIRUS PLAQUE ASSAY
SPLEEN NK ASSAY
PLASMA INTERFERON ASSAY

Figure 1
Figure 2
Figure 3

Figure 4

PATHOGENESIS OF MURINE CYTOMEGALOVIRUS INFECTION
IN NATURAL KILLER CELL-DEPLETED MICE

Running head: NK CELLS IN MURINE CMV PATHOGENESIS

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Departments of Pathology and of Molecular Genetics and Microbiology,
University of Massachusetts Medical Center,
Worcester, Massachusetts 01605
ABSTRACT

The effect of natural killer (NK) cells on the course of acute and persistent murine cytomegalovirus (MCMV) infection was examined by selectively depleting NK cell activity by injection of mice with antibody to asialo GM1, a neutral glycosphingolipid present at high concentrations on NK cells. The dose of MCMV required to cause 50 percent mortality or morbidity in control C57BL/6 mice dropped 4- and greater than 11-fold, respectively, in mice first treated with anti-asialo GM1. NK cell-depleted mice had higher (up to 1000-fold) virus titers in their lungs, spleens, and livers at 3, 5, 7, and 9 days postinfection. Spleens and livers of control mice were virus-free by 7 days postinfection, and their lungs showed no signs of active infection at any time. In contrast, MCMV had disseminated to the lungs of NK cell-depleted mice by day 5, and these mice still had moderate levels of virus in their lungs, spleens, and livers at day 9. Markedly severe pathological changes were noted in the livers and spleens of NK cell-depleted, MCMV-infected mice. These included ballooning degeneration of hepatocytes and spleen necrosis. MCMV-infected NK cell-depleted mice had severe spleen leukopenia, and their spleen leukocytes exhibited a significantly lower (up to 13-fold) response to the T cell mitogen concanavalin A when compared to uninfected or MCMV-infected controls. It appeared that NK cells exerted their most potent antiviral effect early in the infection, in a pattern correlating with IFN production and NK cell activation; treatment with anti-asialo GM1 later in infection had no effect on
virus titers.

The relative effect of NK cell depletion on MCMV pathogenesis depended on the injection route of virus. NK cell depletion greatly augmented MCMV synthesis and pathogenesis in mice inoculated either intravenously or intraperitoneally, but had no effect on the course of disease following intranasal inoculation at any time point examined. One month after i.p. inoculation of virus, NK cell depletion resulted in a 6-8-fold increase in salivary gland virus titers in persistently infected mice, suggesting that NK cells may be important in controlling virus synthesis in the salivary gland during persistent infection. This treatment did not, however, induce dissemination of virus to other organs. These data support the hypothesis that NK cells limit the severity, extent, and duration of acute MCMV infection, and that they may also be involved in regulating the persistent infection.
INTRODUCTION

Human cytomegalovirus (CMV) (reviewed in 33,36,44) is a ubiquitous herpesvirus capable of causing congenital birth defects (13), mononucleosis (24), hepatitis (12), and interstitial pneumonitis (14). It causes acute, persistent, and latent infections, and can be reactivated from a latent state (reviewed in 44). CMV infection is particularly troublesome when patients are immunosuppressed during tumor therapy and transplantation procedures; patients frequently contract CMV pneumonitis, which has a high mortality rate (14). CMV itself is immunosuppressive (46), and the combination of that effect with the effects of chemotherapy and radiation can lead to opportunistic infections such as Pneumocystis carinii (43).

Because of its similarity to human CMV, murine cytomegalovirus (MCMV) (reviewed in 44) infection in mice has been used extensively as a model to study the pathogenesis of and the immune response to CMV. Such studies have shown a peak in natural killer (NK) cell activity 3-5 days postinfection (1,41) and a virus-specific H-2 restricted cytotoxic T lymphocyte (CTL) response 6-20 days postinfection (39). The CTL response is thought to be responsible for the clearance of the virus late in infection (18). However, there are marked differences in the susceptibility of various strains of mice to MCMV (as judged by death 3-5 days postinfection) and resistance correlates with the magnitude of the NK cell rather than CTL response (1). No strain-related differences in macrophage
NK cell-mediated cytotoxicity is augmented during acute (17,31,53,54) and persistent (4) viral infections. This is a result of both proliferation and activation of the NK cell population (2). NK cells can be directly activated by interferon (IFN) (8,9,51,55) or by viral glycoproteins (6). Indirect evidence has supported the concept that NK cells may mediate resistance to herpes simplex virus (30), Friend leukemia virus (26), and mouse hepatitis virus (MHV) (29,50). With regard to MCMV, Shellam and coworkers (1) showed that strains of mice having high NK cell activity were more resistant to MCMV infection than strains having low NK cell activity, and they also observed that NK cell-deficient homozygous beige mice were more susceptible to infection than their heterozygous NK-sufficient littermates (49). Using bone marrow chimeras they demonstrated that bone marrow-derived cells were responsible for this resistance (49); however, since beige mice have other immune defects (52) it was not certain which defect was responsible for the lowered resistance.

There is some suggestive evidence that a lymphocyte may control salivary gland MCMV titers during persistent infection (16), and that a lymphocyte resembling an NK cell, in concert with serum from mice persistently infected with MCMV, can mediate antibody dependent cell-mediated cytotoxicity (ADCC) against MCMV-infected targets (40).

Recently, antibody to asialo GM1, a reagent which selectively depletes NK cell activity in vivo (11,22,23), has become available. While almost totally depleting NK cell activity, it has no effect on
cytotoxic macrophage (23), cytotoxic T (22, 23), or natural cytotoxic (NC) cell (unpublished) functions. In addition, the concanavalin A (Con A) response (11) and the percentage of thy-1 positive (22) and surface immunoglobulin positive (unpublished) cells found in the spleens of in vivo-treated mice are unaffected. It does not react with granulocytes (11), and in the presence of complement lyses less than 5 percent of the total number of spleen leukocytes (22, 25), while eliminating virtually all large granular lymphocytes, which contain the NK cells (25). We have recently shown that mice treated with this antibody synthesize more MCMV, MHV, and vaccinia virus in their livers and spleens and have greater virus-induced liver damage than do untreated mice 3 days postinfection (5). The antibody did not abrogate resistance to infection by inhibiting the IFN response, as antibody-treated MCMV-infected mice had higher levels of plasma IFN than untreated, MCMV-infected mice (5).

The results presented in this paper show that NK cell-depleted mice undergo a more severe and disseminated infection of longer duration than control MCMV-infected mice, and that NK cell depletion is most effective early in infection. The suppression of the T cell response (19) seen in control MCMV-infected mice is greatly enhanced in NK cell-depleted mice, correlating with higher virus titers and delayed viral clearance. Finally, we present some evidence suggesting that NK cells may play a role in controlling persistent MCMV infection.
MATERIALS AND METHODS

Animals. C57BL/6 (+/+, bg/+, and bg/bg) and BALB/c mice were purchased from the Jackson Laboratory, Bar Harbor, ME. Mice of either sex, 6 to 12 wk old, were used in these experiments.

Cells. YAC-1 cells were derived from a Moloney leukemia virus-induced lymphoma in A/Sn mice and were maintained in RPMI 1640 medium (GIBCO, Grand Island, NY) supplemented with antibiotics, glutamine, and 10 percent heat-inactivated fetal bovine serum (FBS) (M.A. Bioproducts, Walkersville, MD). Mouse embryo fibroblasts (MEF) were obtained as described (47) from C57BL/6 mouse embryos, and maintained in minimal essential medium (MEM) (GIBCO) with the same additives as listed above. L-929 cells, a continuous liver cell line derived from C3H mice, were maintained in MEM, as above.

Virus. The Smith strain of MCMV was obtained from Dr. John Nedrud, Case Western Reserve University School of Medicine, Cleveland, OH (35). This virus was maintained by in vivo passage in weanling BALB/c mice. Salivary glands from mice inoculated 2 to 3 wk previously with $10^4$ pfu MCMV were homogenized in a 10 percent suspension and cleared by centrifugation. Aliquots were stored at -70°C in 10 percent DMSO. The Indiana strain of vesicular stomatitis virus (VSV) was used in the interferon assays.

Anti-asialo GM1 antiserum. Rabbit antiserum to asialo GM1 was purchased from Wako Chemicals, USA, Inc., Dallas, TX. This antiserum has previously been shown to deplete NK cell activity in vivo and in vitro (11,22,23) selectively. To deplete NK cell
activity in vivo, anti-asialo GM1 was diluted 1/10 in RPMI medium and given i.v. in a volume of 0.2 ml.

Cytotoxicity assay. Assay medium was RPMI medium supplemented with 0.1 M N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) (Sigma Chemical Company, St. Louis, MO), 10 percent FBS, glutamine, and antibiotics. The assay was performed as described (55). Briefly, YAC-1 target cells, labeled with 100 μCi sodium chromate ($^{51}$Cr; New England Nuclear Corp., Boston, MA) for 1 hr at 37°C, were washed and mixed with various numbers of effector cells in round-bottomed microtiter wells at $10^4$ cells/well. For spontaneous release determination, medium was added to the wells, and 1 percent Nonidet P-40 was added for maximum release determination. Plates were incubated 4 to 16 hr at 37°C in a humidified atmosphere of 5 percent CO$_2$, 95 percent air. At the end of the incubation, plates were centrifuged at 200 x G for 5 min, and 0.1 ml of the supernatant was collected and counted for radioactivity in a Beckman Gamma 5500 counter (Beckman Instruments, Palo Alto, CA). Data are expressed as percent specific release:

$$100 \times \frac{\text{cpm experimental} - \text{cpm spontaneous}}{\text{cpm maximum} - \text{cpm spontaneous}}$$

Spontaneous release was between 8 and 33 percent. Standard deviations of quadruplicate replica samples were less than 10 percent of the mean and were not listed in the tables. Results of cytotoxicity assays against YAC-1 cells using individual mice are tabulated as the mean percent specific $^{51}$Cr release of four separate mice ± SEM.
Virus titration. MCMV titers were determined by using a 10 percent homogenate of tissue taken from individual mice. The number of plaque-forming units (pfu) was determined by plaque assay using MEF and 10-fold dilutions of tissue homogenate. Results are expressed as the geometric mean titer, i.e., the arithmetic averages of the logs of four separate animals titrated for virus individually + SEM. Titers reported are $\log_{10}$ pfu per whole spleen, both lungs, both submaxillary salivary glands, and gram of liver. Where standard errors are omitted, one or more mice in that particular group had no detectable virus in that organ, and these mice were assigned a virus titer equivalent to the lowest amount of virus detectable by our assay method, and averaged together with the other mice in that same group. In this instance, the resulting number was designated "less than." The P values represent the significance of the differences of the means between the designated sample and the normal, non-antibody-treated control, and were calculated using Student's t-test. The mean lethal dose ($LD_{50}$) was calculated by the method of Reed and Meunch (45), using 4 mice per group and serial 2-fold dilutions of MCMV. The mean morbidity dose (morbidity dose$_{50}$) was calculated in the same manner, using ruffled fur and a hunched posture as criteria for clinical illness.

Mitogen stimulation. Spleen cells were dispensed in flat-bottomed microtiter wells at $3 \times 10^5$ cells/well in medium containing 2 $\mu$g/ml concanavalin A (Con A) (Sigma) or medium alone. These cells were incubated in the same manner as those used in cytotoxicity assays for two days, followed by addition of 1 $\mu$Ci $^3$H-thymidine
(New England Nuclear) to each well. The next day, the cells were harvested using a MASH (Belco, Vineland, NJ) and counted in a liquid scintillation counter (Beckman). Spleen cells from at least two mice were pooled, and each number represents the mean counts per minute (cpm) of 4 duplicate wells ± SEM.

Interferon (IFN) assay. Blood was collected in heparinized Natelson tubes from the retro-orbital sinus of mice anesthetized with ether, and plasma was obtained by centrifugation. Peritoneal washes were obtained by injecting mice i.p. with 3 ml cold RPMI medium and collecting the fluid with a Pasteur pipet. This wash was centrifuged at 200 x G for 5 min, and IFN assays were performed on the supernatants. Plasma or peritoneal wash samples were titrated by twofold serial dilutions in a 96-well, flat-bottomed microtiter plate to which L-929 cells were added at 3 x 10⁴ cells/well. Eighteen to 24 hr later, the wells were challenged with 100 TCID₅₀ units of VSV. IFN titers were expressed as the log₂ of the highest reciprocal dilution resulting in 50 percent reduction in cytopathic effect. Results are expressed as the geometric mean titers of four separate animals titrated for IFN individually ± SEM.

Histopathology. Tissue sections were fixed in buffered formalin, embedded in paraffin, cut at 4 μm, and stained with hematoxylin-eosin. An American optical 110 microscope was used for examining tissue sections. A low power field refers to the use of a 10x objective and 10x wide field oculars, yielding a field diameter of 1.7 mm. Tissue sections were examined in a "blind" manner, without knowing whether or not the mouse was NK cell-depleted.
RESULTS

Susceptibility to MCMV infection. When titrated in control C57BL/6 mice, $5 \times 10^5$ pfu of MCMV were equivalent to one LD$_{50}$, but when these mice were treated with anti-asialo GM1 4-6 hours before infection, only $1.2 \times 10^5$ pfu were equivalent to one LD$_{50}$. At a dose of $1.5 \times 10^5$ pfu, 75 percent of antibody-treated mice and 0 percent of control mice died ($P<.01$). Whereas the dose of this stock required to cause 50 percent morbidity (morbidity dose$_{50}$) in control mice was $2 \times 10^5$ pfu, the morbidity dose$_{50}$ in anti-asialo GM1-treated mice was less than $1.8 \times 10^4$ pfu, the lowest dose used in the titration. Further, in other experiments reported throughout this paper, a dose of $5 \times 10^3$ pfu routinely rendered NK cell-deficient mice clinically ill while having no visible effect on control mice. Thus, there were 4- and at least 11-fold decreases in LD$_{50}$ and morbidity dose$_{50}$, respectively, when NK cells were depleted by treatment with anti-asialo GM1.

Antibody treatment did not abrogate resistance to MCMV by inhibiting the IFN response, as both control and antibody-treated mice had similar plasma IFN titers 6 hours after i.p. inoculation with $3 \times 10^5$ pfu MCMV. Control and NK cell-depleted mice had $9.3 \pm 0.2$ and $9.5 \pm 0.3 \log_2$ units IFN/ml plasma, respectively. With regard to the local IFN response, control and NK cell-depleted mice had $2.5 \pm 0.3$ and $3.0 \pm 0.4 \log_2$ units IFN/ml peritoneal wash, respectively. These data, coupled with our previous observation at 3 days postinfection with $5 \times 10^3$ pfu (5), indicate that antibody
treatment did not abrogate the IFN response 6-72 hours postinfection. 

Effect of NK cell depletion on viral synthesis, dissemination, and clearance. Control mice or those treated with anti-asialo GM1 were injected i.p. with 5 x 10^3 pfu MCMV and sacrificed at various times postinfection. The results listed in Table 1 show that NK cell-depleted mice had significantly higher virus titers in the spleen, lung, and liver than control mice at 3, 5, 7, and 9 days postinfection. The magnitude of the differences in virus titer was most pronounced in the spleen, with NK cell-depleted mice having as much as a 1000 times more virus. Though dissemination of virus to the salivary gland was not accelerated in NK cell-depleted mice, an active infection of the lung was noted at 5, 7, and 9 days postinfection only in NK cell-depleted mice (Table 1) with control mice always having fewer than 100 pfu in their total lung tissue. These results indicate that NK cell depletion resulted in increased viral dissemination to the lung.

By nine days postinfection, control mice had no detectable virus in their spleens and livers, while NK cell-depleted mice still had about 5000 pfu/organ (Table 1), indicating that viral clearance from these organs was delayed.

As reported previously (5), NK cell-mediated lysis was almost totally eliminated by anti-asialo GM1 treatment when assayed 3 days postinfection (Table 1). By 5 days postinfection lytic activity was again detectable, and on day 9, NK cell activity on a cell-to-cell basis was comparable to the control.

Under these conditions of infection, control mice did not
become clinically ill (i.e., hunched posture and ruffled fur), and their livers and spleens were completely virus-free by 7-9 days postinfection. In contrast, NK cell-depleted mice were clinically ill 4-9 days postinfection, with moderately high virus titers in the lung, spleen, and liver 9 days postinfection (Table 1), eventually falling to undetectable levels by 12-15 days postinfection (data not shown). In both untreated and antibody-treated mice, MCMV remained in the salivary glands for at least five weeks.

Pathology. As reported earlier (5), livers from NK cell-depleted mice 3 days postinfection had more foci of inflammation than normal MCMV-infected mice as well as large areas of hepatic necrosis (data not shown). Five days postinfection, livers from control MCMV-infected mice had resolving focal hepatitis and were undergoing active regeneration (Fig. 1E). There were many mitotic figures, and the hepatocytes had vesicular cytoplasm and pleomorphic nuclei. The inflammatory foci were small, numbering two per low power field, and were composed predominantly of mononuclear cells with a few admixed neutrophils. Though a few giant cells were seen, cells with viral inclusions were not present. In contrast, livers from NK cell-depleted, MCMV-infected mice had severe and continuing disease, and were yellow and atrophic in appearance (Fig. 1B). The hepatocytes had severe ballooning degeneration with multifocal areas of inflammation, and cells with viral intranuclear inclusions were present (Fig. 1F). No regenerative changes were evident.

Continuing resolution of hepatitis was evident in livers taken from control MCMV-infected mice 9 days postinfection. Regenerative
changes were nearing completion, as livers had fewer mitotic figures and diminished nuclear pleomorphism compared to those examined five days postinfection (Fig. 1G). There was one inflammatory focus per 5 low power fields, and small portal lymphocytic infiltrates were present. Livers taken from NK cell-depleted MCMV-infected mice at 9 days were undergoing active regeneration, but had continuing focal hepatitis which appeared to be in the early stages of resolution (Fig. 1H). There were many mitotic figures, and the hepatocytes had vesicular cytoplasm and pleomorphic nuclei. There were 5 inflammatory foci per low power field, and although there were no viral inclusions, some giant cells were present. In NK cell-depleted, MCMV-infected mice, hepatitis and liver damage at 9 days was more extensive than that seen in control MCMV-infected mice at 5 days postinfection.

Spleens from control MCMV-infected mice 9 days postinfection were larger than those of uninfected mice (Fig. 1A). They had hyperplasia of the white pulp and floridly reactive germinal centers with immunoblasts and plasma cells present in the marginal zones (Fig. 1C). The red pulp was relatively decreased in amount, and contained occasional neutrophils. Cells with viral inclusions were not present. At 9 days, spleens from NK cell-depleted MCMV-infected mice exhibited multifocal necrosis (Fig. 1A). Microscopically, perifollicular necrosis and extensive destruction of the red pulp were seen (Fig. 1D). Multiple small and large thrombi were present in red pulp vessels (Fig. 1D). It is likely that necrosis of the red pulp eventuated in thrombosis of blood vessels with superimposed
infarction of large areas of the spleen. Germinal centers were not present, but there were focal aggregates of plasma cells. Cells with viral inclusions were not present.

Although some lungs examined 5, 7, and 9 days postinfection had mild focal interstitial pneumonitis, there was no difference in lung pathology between NK cell-depleted and control mice.

Effect of NK cell depletion on MCMV-induced immunosuppression.

Previous results have demonstrated that spleen cells from MCMV-infected mice have a diminished response to the T cell mitogen concanavalin A (19). Table 2 confirms these results, and also shows that NK cell depletion enhances this MCMV-induced suppression, while having no effect on the Con A response in uninfected mice. On days 5, 7, and 9 postinfection, mice treated with antibody had Con A responses 13-fold, 5-fold, and 2-fold lower than control MCMV-infected mice, respectively. NK cell depletion also resulted in MCMV-induced leukopenia. Whereas antibody treatment alone had no detectable effect on spleen cell numbers (11, unpublished), anti-asialo GM1-treated MCMV-infected mice had up to 5-fold fewer spleen leukocytes than MCMV-infected controls (Table 3).

Depletion of NK cells on various days postinfection. In order to determine the time interval during which NK cells were exerting their most potent antiviral effects, we treated groups of mice with anti-asialo GM1 on days 0, 1, and 2 postinfection and compared these groups to control MCMV-infected mice; all mice were sacrificed on day 3 postinfection. No significant differences in liver virus titers were seen between the groups whose NK cells were depleted at
0, 1, or 2 days postinfection, but all 3 groups had 25 to 50-fold higher titers than control MCMV-infected mice (Table 4). In slight contrast to the liver data, depletion of NK cells 2 days postinfection failed to enhance spleen virus titers as effectively as depletion at days 0 or 1, but nonetheless significant enhancement of day 3 virus titers was noted in all NK cell-depleted mice. This indicates that the absence of NK cells between days 2-3 and days 1-3 resulted in enhanced day 3 virus titers in the liver and spleen, respectively. This means that NK cells present between 2 and 3 days postinfection were required for the antiviral effect. This period of time corresponds to that necessary for IFN production and activation of NK cells (49). Note that MCMV infection induced higher day 3 plasma IFN titers in anti-asialo GM1-treated mice than in control mice, regardless of whether the antibody was given 0, 1, or 2 days postinfection (Table 4).

We next tested whether NK cell depletion later in infection (day 6, when spleen virus is still detectable) would have any effect on viral titers in the spleen 9 days postinfection. Table 5 shows that the dose of anti-asialo GM1 which totally depleted NK cell activity in our previous experiments (Table 1, day 3) reduced NK cell-mediated lysis by only about 30 percent, when given on day 6 and assayed on day 9 postinfection. It also had no effect on day 9 virus titers in the spleen, liver, or lung. This was in contrast to the data we had obtained when giving the antibody 4 to 6 hours before infection. This was not an unexpected result, since activated NK cells are more resistant to the effects of anti-asialo GM1.
treatment (H. Yang et al., manuscript in preparation). In order to address the possibility that day 9 virus titers were unaffected due to incomplete elimination of NK cells, we gave antibody on 3 separate days (6, 7, and 8) postinfection, or none at all, and sacrificed the mice on day 9. Table 5 shows that most of the NK cell activity was removed by this treatment, but that mice undergoing this treatment had no significant difference in virus titers as compared to controls. This shows that NK cells are not necessary for the elimination of virus that occurs in the spleen between days 6 and 9, nor does NK cell depletion at 6-9 days postinfection bring about dissemination to the lung or reappearance of virus in the liver.

Effect of inoculation route on NK cell-mediated resistance. NK cell depletion had similar effects on MCMV synthesis 3 days postinfection whether the virus was given i.p. or i.v. Table 6 shows that differences as great as 1000-fold were noted in the spleen, and NK cell-depleted mice also had low but detectable virus titers in the lung, which would increase by day 5 (Table 1). These data confirm and extend our previous results (5). NK cell-deficient beige mice yielded similar results (Table 6), in accord with the observation of Shellam et al. (49).

In contrast to the above observations, NK cell depletion had no effect on the course of intranasal infection at 3 days or at later times postinfection (Table 7). During the first 5 days after intranasal MCMV infection, virus synthesis occurs only in the lung (21). NK cell depletion had no effect on lung virus titers at any
time postinfection, nor did it result in accelerated dissemination to the salivary gland (Table 7) or to any other organ tested (spleen, liver, kidney) (data not shown). In fact, dissemination to any organ except the salivary gland was rarely seen, and it did not correlate with the presence or absence of NK cells. **NK cell depletion enhances salivary gland MCMV titers during persistent infection.** To examine the role of NK cells in controlling persistent MCMV infection, we treated mice 30 days postinfection (infectious virus is found only in the salivary gland at this time) with thioglycolate and RPMI or thioglycolate and anti-asialo GM1. The thioglycolate technique was reported by Braughtigam et al. (3) to facilitate rescue of MCMV from latently infected mice. Five days after this treatment (35 days postinfection), the mice were sacrificed; their peritoneal macrophages were cocultivated with MEF, and their spleens, livers, kidneys, blood, lungs, and salivary glands were titrated for virus. MCMV was isolated from all macrophage cultures from both control and NK cell-depleted mice. NK cell depletion had no effect on the rapidity of virus isolations from cocultivated peritoneal macrophages, nor did it result in the reappearance of infectious virus in any of the organs tested (data not shown). However, NK cell depletion resulted in a 6-8-fold increase in salivary gland virus titers in three separate experiments (Table 8). Thus, NK cells may be involved in limiting viral synthesis in the salivary gland during the persistent phase of the infection.
DISCUSSION

The results presented in this paper suggest that NK cells may play a major role in limiting the mortality, morbidity, spleen necrosis, hepatitis, and immunosuppression associated with acute MCMV infection. It is likely that they limit the severity and duration of the infection by controlling viral synthesis and dissemination during the first few days of infection. Our experiments (Tables 4+5) suggest that NK cells may exert their most effective antiviral action at a time correlating with the onset of peak IFN production (49), NK cell activation (49), and NK cell division (2), i.e., between 24 and 72 hours postinfection. Though mice depleted of NK cells 4-6 hours preinfection had elevated virus titers 3-9 days postinfection (Table 1), mice depleted of NK cells 6 days postinfection had no detectable virus in their spleens or livers on day 9 (Table 5), suggesting that NK cells may not be necessary for viral clearance later in the course of acute infection, when MCMV-specific T cells are present. Clearance of virus later in infection has been shown by Ho (18) to be a function of H-2 restricted MCMV-specific T cells.

NK cell depletion before MCMV infection results in a 500 to 1000-fold increase in spleen virus titers 3 days postinfection, before CTL are detectable. The delayed clearance of virus seen 9 days postinfection in NK cell-depleted mice may simply be due to the large viral antigen load resulting from NK cell depletion early in the infection, or it could also be due to increased virus-induced
suppression of T cell function. It is well known that MCMV can suppress T cell function (37,19), and that the degree of suppression correlates with virus titers (19). In our study, mice depleted of NK cells had higher virus titers and a greater degree of MCMV-induced suppression of the Con A response (Table 2) as compared to controls. Since the Con A response is a measure of T cell-dependent function, it is likely that viral clearance, also a T cell-dependent function, could be inhibited, resulting in an infection and illness of longer duration.

The consequences of NK cell depletion on the pathogenesis of MCMV infection were profound. Severe ballooning degeneration of hepatocytes has previously been reported in irradiated, MCMV-infected mice (15), but due to the lack of specificity of irradiation, the immune deficit responsible for this was not determined. Mims and Gould (34) reported spleen necrosis (but not thrombosis and infarction) in MCMV-infected mice. However, even when high inoculating doses are given, mouse strains with genetically high NK cell activity (CBA, C57BL/6, and C57BL/10) (38) do not present with spleen necrosis, whereas strains with genetically low NK cell activity (A, BALB/c) (38) have necrotic spleens (34). Our experiments confirm and extend this correlation, showing that NK cell depletion of C57BL/6 mice followed by a low dose (5 x 10^3 pfu) MCMV infection resulted in spleen necrosis.

The absence of NK cells also resulted in dissemination of the virus to the lung. This condition, coupled with immunosuppression, could set the stage for opportunistic infection in humans.
Immunocompromised cancer and transplant patients, whose NK cell activity is low, are susceptible to CMV pneumonitis (42). Almost all patients with acquired immunodeficiency syndrome (AIDS) have active CMV infection (27), and though the evidence for CMV as a cause for AIDS is not convincing, it is certainly possible that lowered NK cell activity could allow CMV to flourish and to exert its immunosuppressive effects, establishing optimal conditions for a putative AIDS-causing agent or other opportunistic agents to take over.

Whereas NK cell depletion had a dramatic effect on the course of i.p. or i.v. MCMV infection, it had no detectable effect on the course of intranasal infection. NK cells are present in the lung (41), and this laboratory has shown that antibody to asialo GM1 depletes lung NK cell activity and prevents the rejection of NK-sensitive YAC-1 cells from the lung (C. Biron, K. Okumura, S. Habu, and R. Welsh, J. Virol. in press, JVI 608). Quinnan et. al. (41) have shown that cortisone treatment increases lung MCMV titers during the first 7 days of intranasal infection, and they suggested that this increase was due to the depletion of lung NK cells they observed. Our present results indicate that NK cells are not mediating resistance during intranasal infection, and that some other cortisone-sensitive effector system (not T cells) (41) is responsible for resistance against MCMV in the lung. In support of this concept, Biron et. al. (J. Virol. in press, JVI 608) have demonstrated that a cortisone-sensitive non-NK cell is responsible for preferential rejection from the lungs of virus-infected cells.
over uninfected cells in 4 hour in vivo cytotoxicity assays.

A recent report (10) had suggested that early IFN production, 6 hours postinfection, mediated resistance to MCMV infection either directly, or via NK cell activation. Our present data show that NK cell depletion with anti-asialo GM1 did not abrogate the early IFN response. Therefore, lack of IFN cannot account for the lowered resistance in the antibody-treated mice.

When administered in vivo, anti-asialo GM1 antibody has no detectable effect on any immune functions tested other than NK cell activity (11,22,23). However, it does bind to some monocytes and thymocytes (11), and it could possibly have unknown effects on non-lymphoid tissue. Recent work in our laboratory (J. Bukowski and R. Welsh, unpublished) provides further evidence that it is indeed NK cells which are mediating resistance. Athymic nude mice treated with anti-asialo GM1 have enhanced MCMV titers. Newborn mice (which are NK cell-deficient) which received adoptively transferred control adult spleen cells resist lethal MCMV infection. The adult cells still protected if they were immunochemically depleted of T cells or depleted of macrophages and granulocytes by size separations. Adult cells did not protect if NK cells were removed by antibody to asialo GM1. Selgrade and Osborn (48), using the same adoptive transfer system, also demonstrated that macrophage-depleted adult spleen cells could mediate protection. A potential mechanism for NK cells to control MCMV infection is also seen from our unpublished results showing that in the presence of IFN, which protects uninfected targets, MCMV-infected cells are much more susceptible to NK
cell-mediated lysis.

MCMV has the capacity to establish persistent and latent infections, which can be reactivated under conditions of immunosuppression (20,32), even in the face of moderately high antibody titers. While we have so far not demonstrated a role for NK cells in controlling reactivation of latent infection, we have shown that NK cell depletion during the persistent phase of the infection results in significant increases in salivary gland virus titers (Table 8), but not dissemination of the virus from that organ. To our knowledge, this is the first body of evidence in support of a role for NK cells in limiting viral synthesis in a persistent viral infection.

It thus appears that NK cells may play a role in limiting viral synthesis and virus-induced pathology in the early stages of some acute viral infections, but not others, such as LCMV (5). The present data suggest that NK cells act early in acute MCMV infection to reduce the viral load at a time period corresponding to IFN production and activation of NK cell-mediated cytotoxicity, but before virus-specific antibody and cytotoxic T cells are detectable. An increased viral load in NK cell-depleted mice could lead to dissemination of virus to the lung. More virus leads to enhanced suppression of the T cell response which could likely result in delayed clearance of virus, and a prolongation of hepatitis. It thus appears likely that NK cells acting early in the infection can limit the severity, extent, and duration of acute MCMV infection, and that they may play a role in controlling persistent
infection.
ACKNOWLEDGEMENTS

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We thank Eva Moring for preparation of the tissue sections and Chris Hebert for photographing the specimens and processing the color photographs.
LITERATURE CITED


TABLE 1

NATURAL KILLER CELL DEPLETION RESULTS IN INCREASED VIRAL DISSEMINATION AND DELAYED CLEARANCE OF VIRUS

<table>
<thead>
<tr>
<th>days post-anti-infection</th>
<th>AGM1</th>
<th>NK lysis</th>
<th>percent</th>
<th>log_{10} pfu/</th>
<th>spleen</th>
<th>lung</th>
<th>g. liver</th>
<th>sal. gland</th>
</tr>
</thead>
<tbody>
<tr>
<td>3^a</td>
<td>-</td>
<td>20. +1.6</td>
<td>1.7 + 0.2</td>
<td>&lt;1.0^c</td>
<td>3.1 + 0.1</td>
<td>&lt;2.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>0.1 + 0.9</td>
<td>4.6 + 0.1^b</td>
<td>1.6 + 0.1</td>
<td>4.7 + 0.1^b</td>
<td>&lt;2.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>-</td>
<td>12. +1.2</td>
<td>2.0 + 0.1</td>
<td>1.5 + 0.1</td>
<td>&lt;2.8</td>
<td>&lt;2.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>8.5 + 1.6</td>
<td>4.3 + 0.1^b</td>
<td>3.9 + 0.0</td>
<td>4.7 + 0.1^b</td>
<td>&lt;2.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>-</td>
<td>13. +2.0</td>
<td>&lt;1.9</td>
<td>&lt;1.1</td>
<td>&lt;2.4</td>
<td>2.9 + 0.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>8.6 + 0.4</td>
<td>3.7 + 0.2^b</td>
<td>2.9 + 0.1</td>
<td>3.4 + 0.4^b</td>
<td>3.4 + 0.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>-</td>
<td>8.5 + 1.2</td>
<td>&lt;0.7</td>
<td>1.5 + 0.3</td>
<td>&lt;2.5</td>
<td>5.3 + 0.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>6.5 + 0.7</td>
<td>3.7 + 0.3^b</td>
<td>3.4 + 0.1</td>
<td>3.1 + 0.1^b</td>
<td>5.5 + 0.1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
TABLE 1, continued

Mice were either left untreated or given anti-asialo GM1 (anti-AGM1) 4-6 hrs before i.p. injection of $5 \times 10^3$ pfu MCMV. Four C57BL/6 mice in each treatment group were sacrificed at various times postinfection; virus titrations and NK cell assays were performed. The assay length was 16 hrs, and the effector to target ratio was 11:1.

Where numbers are preceded by a "less than" symbol ($<$), one or more mice had no detectable virus in that organ (see Materials and Methods).
\begin{table}
\centering
\begin{tabular}{lcccc}
\hline
& days postinfection & 3 & 5 & 7 & 9 \\
\hline
\textbf{Uninfected} & & & & & \\
unstimulated & 5.3 ± 1.0 & 5.6 ± 1.1 & 9.4 ± 1.2 & 7.9 ± 0.7 \\
Con A & 1200. ± 56. & 1400. ± 14. & 690. ± 16. & 1000. ± 22. \\
\textbf{Uninfected + anti-AGM1} & & & & & \\
unstimulated & 8.6 ± 0.6 & 6.1 ± 0.4 & ND & 7.4 ± 0.4 \\
Con A & 1200. ± 94. & 1400. ± 24. & ND & 1100. ± 19. \\
\textbf{MCMV-infected} & & & & & \\
unstimulated & 22. ± 2.5 & 2.7 ± 0.3 & 12. ± 2.0 & 9.7 ± 0.5 \\
Con A & 1200. ± 79. & 490. ± 19. & 310. ± 14. & 1000. ± 11. \\
\textbf{MCMV-infected + anti-AGM1} & & & & & \\
unstimulated & 15. ± 2.6 & 41. ± 3.2 & 21. ± 2.9 & 15. ± 1.2 \\
Con A & 830. ± 25. & 36. ± 1.8 & 60. ± 3.3 & 540. ± 6.2 \\
\hline
\end{tabular}
\caption{NK CELL DEPLETION RESULTS IN INCREASED MCMV-INDUCED SUPPRESSION OF THE CON A RESPONSE\textsuperscript{a}}
\end{table}
TABLE 2, continued

aMice were left untreated or given anti-asialo GM1 (anti-AGM1). Four to six hr later, half of each group was infected i.p. with 5 x 10^3 pfu MCMV and the other half was left untreated. Mice were sacrificed at various days postinfection and the spleen cells were assayed for response to Con A stimulation as described in Materials and Methods. Each time point represents a separate assay with its own control from uninfected mice. Results are expressed in counts per minute (x 10^2) + SEM.

bND, not done.
**TABLE 4**

**NK CELL DEPLETION ON DAYS 0, 1, OR 2 POST INFECTION RESULTS IN ENHANCED MCMV TITERS ON DAY 3 POST INFECTION**

<table>
<thead>
<tr>
<th>anti-AGM1 percent</th>
<th>log_{10} pfu/ spleen</th>
<th>log_{2} IFN U/ml plasma</th>
</tr>
</thead>
<tbody>
<tr>
<td>on day: NK lysis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0^a</td>
<td>3.7±0.4</td>
<td>3.6±0.1</td>
</tr>
<tr>
<td>1</td>
<td>4.5±0.6</td>
<td>3.3±0.2</td>
</tr>
<tr>
<td>2</td>
<td>5.8±0.6</td>
<td>1.9±0.1</td>
</tr>
<tr>
<td>control</td>
<td>58.±2.3</td>
<td>&lt;1.0^b</td>
</tr>
</tbody>
</table>
Sixteen mice were injected i.p. with $5 \times 10^3$ pfu MCMV on day 0 and divided into groups of 4. One group was left untreated and the other 3 groups were given anti-AGM1 0, 1, or 2 days postinfection. All mice were sacrificed on day 3 postinfection for determination of spleen NK cell activity, virus titers, and IFN titers.

See legend to Table 1, footnote c.
# TABLE 5

NK CELL DEPLETION AT 6 TO 9 DAYS POSTINFECTION HAS NO EFFECT ON DAY 9 MCMV TITERS

<table>
<thead>
<tr>
<th>Exp</th>
<th>AGM1</th>
<th>NK lysis</th>
<th>spleen</th>
<th>lung</th>
<th>g. liver</th>
<th>sal. gland</th>
</tr>
</thead>
<tbody>
<tr>
<td>1^a</td>
<td>-</td>
<td>52. +0.4</td>
<td>&lt;1.3^c</td>
<td>&lt;1.5</td>
<td>&lt;2.5</td>
<td>5.1±0.1</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>35. +3.5</td>
<td>&lt;1.3</td>
<td>&lt;1.5</td>
<td>&lt;2.5</td>
<td>5.2±0.1</td>
</tr>
<tr>
<td>2^b</td>
<td>-</td>
<td>39. +0.3</td>
<td>&lt;1.3</td>
<td>1.5±0.1</td>
<td>&lt;2.5</td>
<td>4.8±0.1</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>5.0±1.0</td>
<td>&lt;1.3</td>
<td>1.5±0.0</td>
<td>&lt;2.5</td>
<td>4.6±0.1</td>
</tr>
</tbody>
</table>
TABLE 5, continued

Two groups of 4 mice were injected i.p. with $5 \times 10^3$ pfu MCMV.
On day 6 postinfection; when 200-500 pfu virus are present in the
spleen, one group was given anti-asialo GM1 (anti-AGM1) i.v. On day
9, the mice were sacrificed, and virus titrations and NK cell assays
were performed.

Same as exp 1, except mice were given anti-AGM1 daily on days 6-8.

See legend to Table 1, footnote c.
TABLE 6

NK CELL DEPLETION RESULTS IN ENHANCEMENT OF MCMV SYNTHESIS WHEN INOCULATION IS I.P. OR I.V.

<table>
<thead>
<tr>
<th>Exp infection</th>
<th>route of infection</th>
<th>AGM1</th>
<th>NK lysis</th>
<th>percent</th>
<th>log$_{10}$pfu/lung</th>
<th>log$_{10}$pfuspleen</th>
</tr>
</thead>
<tbody>
<tr>
<td>1$^a$</td>
<td>i.v.</td>
<td>C57BL/6 (bg/+)</td>
<td>-</td>
<td>61. +4.3</td>
<td>ND</td>
<td>2.3+0.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(bg/bg)</td>
<td>-</td>
<td>47. +4.0</td>
<td>ND</td>
<td>4.4+0.2$^b$</td>
</tr>
<tr>
<td>2</td>
<td>i.v.</td>
<td>C57BL/6</td>
<td>-</td>
<td>ND</td>
<td>&lt;1.0$^d$</td>
<td>1.6+0.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>+</td>
<td>ND</td>
<td>1.7+0.0</td>
<td>4.9+0.0$^b$</td>
</tr>
<tr>
<td>3$^c$</td>
<td>i.p.</td>
<td>C57BL/6 (bg/+)</td>
<td>-</td>
<td>51. +6.1</td>
<td>ND</td>
<td>1.4+0.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(bg/bg)</td>
<td>-</td>
<td>3.8+1.1</td>
<td>ND</td>
<td>4.1+0.1$^b$</td>
</tr>
<tr>
<td>4</td>
<td>i.p.</td>
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<td>-</td>
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<td>&lt;1.0</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>+</td>
<td>0.2+0.9</td>
<td>1.6+0.1</td>
<td>4.6+0.1$^b$</td>
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</table>
TABLE 6, continued

Groups of 4 mice either untreated or given anti-AGM1 were injected either i.p. or i.v. with $5 \times 10^3$ pfu MCMV 4-6 hrs later. All animals were sacrificed on day 3 postinfection, and virus titers were determined.

$^a$ Groups of 4 mice either untreated or given anti-AGM1 were injected either i.p. or i.v. with $5 \times 10^3$ pfu MCMV 4-6 hrs later. All animals were sacrificed on day 3 postinfection, and virus titers were determined.

$^b$ P<0.001.

$^c$ The data in this experiment were published previously (5) and included in this table for comparison.

$^d$ See legend to Table 1, footnote c.
TABLE 7

NK CELL DEPLETION HAS NO EFFECT ON MCMV SYNTHESIS IN THE LUNG AFTER INTRANASAL INOCULATION

<table>
<thead>
<tr>
<th>days post-infection</th>
<th>anti- mouse</th>
<th>AGM1 percent</th>
<th>log_{10} pfu/lung</th>
<th>log_{10} pfu/sal. gland</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 (^a) C57BL/6 (bg/+)</td>
<td>-</td>
<td>45. (\pm) 4.0</td>
<td>3.1 (\pm) 0.4</td>
<td>(&lt;2.0^b)</td>
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<tr>
<td>(bg/bg)</td>
<td>-</td>
<td>7.2 (\pm) 1.4</td>
<td>3.0 (\pm) 0.1</td>
<td>(&lt;2.0)</td>
</tr>
<tr>
<td>C57BL/6</td>
<td>-</td>
<td>63. (\pm) 1.4</td>
<td>3.1 (\pm) 0.1</td>
<td>(&lt;2.0)</td>
</tr>
<tr>
<td>+</td>
<td>0.9 (\pm) 0.4</td>
<td>3.4 (\pm) 0.1</td>
<td>(&lt;2.0)</td>
<td></td>
</tr>
<tr>
<td>5 C57BL/6 (bg+)</td>
<td>-</td>
<td>37. (\pm) 4.3</td>
<td>4.3 (\pm) 0.1</td>
<td>(&lt;2.0)</td>
</tr>
<tr>
<td>(bg/bg)</td>
<td>-</td>
<td>4.1 (\pm) 0.9</td>
<td>4.1 (\pm) 0.2</td>
<td>(&lt;2.0)</td>
</tr>
<tr>
<td>7 C57BL/6</td>
<td>-</td>
<td>68. (\pm) 2.4</td>
<td>4.1 (\pm) 0.1</td>
<td>2.8 (\pm) 0.2</td>
</tr>
<tr>
<td>+</td>
<td>37. (\pm) 8.2</td>
<td>4.3 (\pm) 0.2</td>
<td>2.9 (\pm) 0.3</td>
<td></td>
</tr>
<tr>
<td>9 C57BL/6</td>
<td>-</td>
<td>26. (\pm) 7.3</td>
<td>5.3 (\pm) 0.2</td>
<td>5.1 (\pm) 0.3</td>
</tr>
<tr>
<td>+</td>
<td>14. (\pm) 4.2</td>
<td>5.4 (\pm) 0.1</td>
<td>5.3 (\pm) 0.2</td>
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</tr>
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</table>
Groups of 4 mice either untreated or given anti-AGM1 were inoculated intranasally with $10^5$ pfu MCMV 4-6 hrs later. Mice were sacrificed at various times postinfection; virus titers and NK cell activities were determined.

See legend to Table 1, footnote c.
TABLE 8

NK CELL DEPLETION ENHANCES MCMV TITERS IN PERSISTENTLY INFECTED MICE

<table>
<thead>
<tr>
<th>Exp.</th>
<th>Treatment</th>
<th>percent</th>
<th>( \log_{10}\text{pfu/} )</th>
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<td>saliv. gland</td>
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<td>RPMI</td>
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<td>4.1+0.3</td>
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<td>Anti-AGM1</td>
<td>2.3+0.2</td>
<td>5.0+0.0\text{\textsuperscript{b}}</td>
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<tr>
<td>2</td>
<td>RPMI</td>
<td>31. +2.8</td>
<td>2.9+0.3</td>
</tr>
<tr>
<td></td>
<td>Anti-AGM1</td>
<td>-1.6+0.4</td>
<td>3.8+0.4\text{\textsuperscript{c}}</td>
</tr>
<tr>
<td>3</td>
<td>RPMI</td>
<td>37. +3.4</td>
<td>4.4+0.2</td>
</tr>
<tr>
<td></td>
<td>Anti-AGM1</td>
<td>14. +4.0</td>
<td>5.2+0.1\text{\textsuperscript{b}}</td>
</tr>
</tbody>
</table>
TABLE 8, continued

*Mice were injected i.p with $5 \times 10^3$ pfu MCMV. Thirty days later, groups of 5 mice were injected with either RPMI medium or anti-asialo GM1 (Anti-AGM1), followed by 3 ml 3 percent thioglycolate broth i.p. These mice were sacrificed 5 days later and their organs were titrated for virus; spleen NK cell activity was also determined.

$^aP<.001$

$^bP<.001$

$^cP<.01$
FIGURE LEGEND

Figure 1.

A) Spleens from control MCMV-infected (1), control uninfected (2), or anti-asialo GM1-treated, MCMV-infected (3) mice. The spleen from the control infected mouse (1) is hyperplastic. The spleen from the anti-asialo GM1-treated, MCMV-infected mouse (3) shows extensive infarction. Bar = 0.38 cm.

B) Liver lobes from control MCMV-infected (1), control uninfected (2), or anti-asialo GM1-treated, MCMV-infected (3) mice. The liver lobe from the anti-asialo GM1-treated, MCMV-infected mouse (3) is smaller and pale in color as compared to the control MCMV-infected (1) or control uninfected (2) mouse. Bar = 0.40 cm.

C) Spleen from control MCMV-infected mouse showing hyperplastic white pulp and intact red pulp. Bar = 100 μm.

D) Spleen from anti-asialo GM1-treated, MCMV-infected mouse with inactive white pulp and necrotic red pulp. Inset shows thromboembolus (arrow) in a splenic vein. Bar = 100 μm.

E) Liver from control mouse 5 days after MCMV infection shows regeneration. The hepatocytes have pleomorphic nuclei and mitoses are frequent (arrows). Few inflammatory cells are seen. Bar = 50 μm.

F) Liver from anti-asialo GM1-treated mouse 5 days after MCMV infection. The hepatocytes show severe ballooning degeneration.
Hepatocytes with viral intranuclear inclusions are present (arrows). Inflammatory cells are sprinkled throughout the liver and regenerative changes are not yet apparent. Bar = 50 μm.

G) Liver from control mouse 9 days after MCMV infection. Continuing resolution of the hepatitis is evident. The hepatocyte nuclei remain somewhat pleomorphic and binucleate cells are frequent. A few inflammatory cells are present in a portal triad (arrow). Bar = 50 μm.

H) Liver from anti-asialo GM1-treated mouse 9 days after MCMV infection shows resolving hepatitis. The hepatocyte nuclei are somewhat pleomorphic. The ballooning degeneration has resolved. Foci of inflammatory cells still remain. A liver giant cell is present (arrow). Bar = 50 μm.
INTERFERON ENHANCES THE SUSCEPTIBILITY OF VIRUS-INFECTED FIBROBLASTS TO CYTOTOXIC T CELLS

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Abbreviations

IFN  interferon
NK   natural killer
CTL  cytotoxic T lymphocyte
MEF  mouse embryonic fibroblast
VV   vaccinia virus
LCMV lymphocytic choriomeningitis virus
Introduction

Interferon (IFN) induced during virus infection may limit disease by direct mediation of antiviral effects in cells or by modulation of the host response. IFN induces the activation and proliferation of natural killer (NK) cells (1-3), enhances macrophage-mediated phagocytosis (4) and cytotoxicity (5), and may be required for the generation of cytotoxic T cells (CTL) (6). IFN also alters the membrane of target cells, rendering them resistant to NK cell-mediated lysis (7) and inducing the expression of cell surface proteins, including major histocompatibility (MHC) antigens (8,9). CTL recognize viral antigens in the context of syngeneic class I MHC antigens (10). It is thus possible, though never shown, that IFN may condition target cells for increased susceptibility to antiviral CTL by inducing MHC antigen expression. We show here that IFN greatly enhances the susceptibility of virus-infected mouse embryonic fibroblasts (MEF) to CTL-mediated lysis and that this correlates with increased expression of mouse MHC (H-2) antigens.
Materials and Methods

Animals. C57BL/6 and BALB/c mice were purchased from the Jackson Laboratories, Bar Harbor, ME. C3H/St mice were purchased from West Seneca Laboratories, West Seneca, NY. Mice of either sex, 6 to 16 weeks old were used in these experiments.

Cells. Mouse embryonic fibroblasts (MEF) from BALB/c (H-2^d^) or C57BL/6 (H-2^b^) mice were prepared as described (16), and maintained in minimal essential medium (MEM) (GIBCO, Grand Island, NY) supplemented with antibiotics, glutamine, and 10 percent heat-inactivated fetal bovine serum (FBS) (M.A. Bioproducts, Walkersville, MD). The continuous cell lines L-929 (H-2^k^) and MC57G (H-2^b^) were also maintained on MEM. BHK cells were grown in Dulbecco's MEM with 10% tryptose phosphate broth as an additive.

Viruses. Lymphocytic choriomeningitis virus (LCMV), Armstrong strain, was grown in BHK cells. Vaccinia virus (VV), strain WR, was grown in mouse L-929 cells.

Treatment of target cells. Target cells were dispensed in 60 mm Petrie dishes and some were infected with LCMV at an m.o.i. of 0.05 and incubated for 2 days. VV was added at an m.o.i. of 5.0 and the incubation period was 10 hr. IFN-β (Lee Biomolecular, San Diego, CA) was then added to some of the virus-infected and uninfected cultures at 10,000 U/ml, and the incubation was continued for an additional 12-24 hrs. In some experiments, supernatants containing 64 U/ml IFN-γ generated from concanavalin A-stimulated spleen cells were added. The cells were then used as targets in cytotoxicity assays.

Cytotoxicity assay. Assay medium was RPMI-1640 medium supplemented with 0.1 M N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) (Sigma Chemical
Co., St. Louis, MO), 10% FBS, glutamine, and antibiotics. The assay was performed as described (1). Briefly, target cells labeled with 100 uCi sodium chromate (New England Nuclear, Boston, MA) for 1 hr at 37°C were washed and mixed with various numbers of effector cells in round-bottomed microtiter wells at $10^4$ target cells/well. For spontaneous release determination, medium was added to the wells, and 1% Nonidet P-40 was added for maximum release determination. Plates were incubated for 6-8 hrs. at 37°C in a humidified atmosphere of 5% CO$_2$, 95% air. At the end of the incubation, plates were centrifuged at 200 x g for 5 min, and 0.1 ml of the supernatant was collected and counted for radioactivity in a Beckman Gamma 5500 Counter (Beckman Instruments, Palo Alto, CA). Data are expressed as percent specific release:

$$100 \times \frac{\text{cpm experimental} - \text{cpm spontaneous}}{\text{cpm maximum} - \text{cpm spontaneous}}$$

Spontaneous release was between 16 and 28%. Standard deviations of quadruplicate replica samples were less than 10% of the mean, and are not shown.

**Quantitation of cell surface antigens.** For analysis of LCMV surface antigens, $5 \times 10^5$ target cells were treated with mouse anti-LCMV antiserum at a final dilution of 1:50 in a volume of 150 ul, and incubated for 45 min at 4°C. The cells were washed and then treated with 100 ul fluorescein isothiocyanate (FITC) - conjugated goat anti-mouse immunoglobulin (Cappell Laboratories, Cochranville, PA) at 1:40 for 45 min at 4°C. The cells were washed and subjected to analysis by flow cytometry. Surface H-2 antigens were quantitated by the same method, using congenic anti-H-2$^b$ (B10. A anti-B10) or anti-H-2$^d$ (B10 anti-B10.D2) antisera obtained from Dr. Rolf Kiessling, Karolinska Institute, Stockholm, Sweden, and used at a final dilution of 1:60.
Results and Discussion

C57BL/6 (H-2b), C3H/St (H-2\textsuperscript{k}), or BALB/c (H-2\textsuperscript{d}) mice were inoculated i.p. with 10\textsuperscript{7} p.f.u. of vaccinia virus (VV) strain WR or 8 x 10\textsuperscript{4} p.f.u. of lymphocytic choriomeningitis virus (LCMV) strain Armstrong. Seven days later, the mice were sacrificed and their spleen leukocytes were used as CTL effectors in cytotoxicity assays. The target cells were low-passage C57BL/6 or BALB/c MEF, or the continuous cell lines, L-929 (H-2\textsuperscript{k}) and MC57G (H-2\textsuperscript{b}). Cells were either untreated, treated with IFN, infected with VV or LCMV, or infected and later treated with IFN as described in Materials and Methods. After labeling with Na\textsuperscript{51}CrO\textsubscript{4}, they were used as targets for CTL. The results in Figure 1 A, B, C indicate that pretreatment of VV- or LCMV-infected MEF with 10,000 U/ml IFN-\textgamma resulted in a substantial increase in the sensitivity of these targets to virus-specific lysis by CTL. IFN-\textgamma did not enhance the low levels of lysis observed with uninfected cells. Pretreatment with supernatants containing 64 U/ml IFN-\textgamma generated from concanavalin A-stimulated spleen cells yielded virtually identical results (data not shown). Lysis of the virus-infected IFN-\textgamma-treated targets was mediated by CTL, as it was H-2 restricted and eliminated by pretreating the effectors with monoclonal anti-thy 1.2 antibody and complement (data not shown). In contrast to the results with T cell killing, IFN-\textgamma induced protection of both uninfected and LCMV-infected target cells against lysis by activated NK cells (Fig. 1 D, H). This demonstration of IFN-mediated protection against NK cells indicates that IFN was not increasing target cell sensitivity to lysis in general. Further, IFN-treated cells did not exhibit greater spontaneous release of label, and they were equally resistant to anti-LCMV antibody plus complement-mediated lysis as compared to controls (data not shown).
IFN-induced enhancement of sensitivity to lysis by CTL was seen with both C57BL/6 and BALB/c MEF, suggesting that this phenomenon may be a general property of low-passage MEF (Fig. 1 A, B, C). However, IFN-α pretreatment of virus-infected continuous cell lines, i.e. LCMV-infected L-929 (Fig. 1 E), VV-infected L-929 (data not shown), LCMV-infected MC57G (Fig. 1 F), VV-infected MC57G (Fig. 1 G), did not enhance their sensitivity to lysis by CTL. This was not an unexpected result, as these targets are already highly sensitive to lysis by CTL.

The structures recognized by virus-specific CTL are virus-induced surface proteins in association with class I MHC antigens (10). IFN is known to enhance the cell surface expression of both MHC (8,9) and certain viral antigens (11) in some systems. To investigate the possibility that IFN was altering surface antigen expression on MEF, we quantitated surface H-2 and LCMV antigens by treating MEF with fluorescein-labeled antibodies and analyzing the cells by flow cytometry. The results show that IFN-α pretreatment of MEF had no significant effect on the expression of surface LCMV antigens (Fig. 2 A), but that both uninfected and LCMV-infected MEF had substantial increases in surface H-2 expression (Fig. 3 A, C, F). Analysis of the continuous cell line MC57G also showed that surface expression of LCMV antigens remained unchanged after IFN pretreatment (Fig. 2 B), but in contrast to the MEF, MC57G cells had similar levels of surface H-2 antigens whether or not they were pretreated with IFN-α (Fig. 3 B, D). Infection with LCMV had only a minor effect on this observation. MC57G cells were sensitive to other IFN-mediated effects, as IFN protected these cells from NK cell-mediated lysis (Fig. 1 H). The level of surface H-2 on the untreated MC57G cells was similar to that on IFN-treated MEF, suggesting that MC57G cells may already express a level of surface
H-2 antigens high enough for efficient association with viral antigens to be good targets for CTL. Treatment of MEF (Fig. 3 E) or MC57G cells (data not shown) with supernatants containing 64 U/ml IFN-γ generated from concanavalin A-stimulated spleen cells yielded nearly identical results.

Since MHC class I-restricted virus-specific T cells are known to eliminate virus in vivo (12), it is possible that IFN enhances this process by increasing the sensitivity of virus-infected cells to lysis by CTL. The source of the IFN could be either virus-induced IFN-α/β or IFN-γ produced by T cells upon recognition of a target (13). T cell-produced IFN-γ may locally enhance MHC expression in focal areas of infection, thereby increasing the sensitivity of T cell recognition of virus-infected tissue. Recent findings by Pfau and his associates (14) show that IFN treatment of mice infected intracranially with LCMV leads to increased mortality. Since death in this system has been shown to be caused by virus-specific T cell-dependent destruction of brain tissue (15), it is possible that IFN may be enhancing H-2 antigen expression on the surface of brain tissue leading to more destruction of LCMV-infected cells by virus-specific T cells. This IFN-induced enhancement of MHC antigens on virus-infected cells may thus possibly augment T cell-dependent immunopathology as well as T cell-dependent clearance of virus.

We thank Ms. Marcia McFadden for performing the flow cytometry, and Ms. Dottie Walsh for typing the manuscript.
Summary

Interferon (IFN) pretreatment of low-passage mouse embryonic fibroblasts (MEF) infected with lymphocytic choriomeningitis virus or vaccinia virus rendered these cells 2 to 3 times more susceptible to lysis by H-2 restricted virus-specific cytotoxic T lymphocytes (CTL) than control, virus-infected MEF. The increased sensitivity to lysis correlated with increased expression of surface H-2 antigens, but not viral antigens. Continuous cell lines already highly sensitive to CTL-mediated lysis and already expressing high levels of surface H-2 antigens were unaffected by IFN pretreatment. These results suggest that IFN treatment, by increasing surface H-2 levels, may result in increased association of surface H-2 and virus antigens, which could lead to enhanced recognition and lysis by virus-specific CTL.
References


**Figure Legends**

**Figure 1.** Enhancement of LCMV- and VV-specific T cell-mediated lysis by pre-treatment of MEF with IFN. •—• virus-infected targets. •—• virus-infected targets pretreated with IFN-β. o—o uninfected targets. o—o uninfected targets pretreated with IFN-β. A. BALB/c day 7 LCMV-immune spleen cells were used as CTL effectors against LCMV-infected or uninfected BALB/c MEF. B. Same as A, except CTL were C57BL/6 and targets were C57BL/6 MEF. C. C57BL/6 day 7 VV-immune spleen cells were used as effectors against VV-infected or uninfected C57BL/6 MEF. D. C57BL/6 spleen cells 3 days after LCMV-infection were used as a source of NK cell effectors (1) against LCMV-infected or uninfected C57BL/6 MEF. E. C3H/St day 7 LCMV-immune spleen cells were used as CTL effectors against LCMV-infected or uninfected L-929 cells. F. C57BL/6 day 7 LCMV-immune spleen cells were used as CTL effectors against LCMV-infected or uninfected MC57G target cells. G. Same as C, except targets were MC57G. H. Same as D, except targets were MC57G.

**Figure 2.** Surface expression of LCMV antigens. A. LCMV-infected C57BL/6 MEF. B. LCMV-infected MC57G.

**Figure 3.** Surface expression of H-2 antigens. A. LCMV-infected C57BL/6 MEF. B. LCMV-infected MC57G. C. Uninfected C57BL/6 MEF. D. Uninfected MC57G. E. Uninfected C57BL/6 MEF. F. Uninfected BALB/c MEF.
Figure 1
Figure 2
Figure 3
ADOPTIVE TRANSFER STUDIES DEMONSTRATING THE ANTIVIRAL EFFECT OF NATURAL KILLER CELLS IN VIVO

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\textsuperscript{2}University of Southern California Comprehensive Cancer Center 2025 Zonal Avenue, Los Angeles, California 90033
Running Title: NK Cells Mediate Antiviral Effects In Vivo
This research was supported by U.S. Public Health Research Grants AI-17672 and CA 34461.

Abbreviations:

<table>
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<th>Abbreviation</th>
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<tr>
<td>IFN</td>
<td>interferon</td>
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<tr>
<td>NK</td>
<td>natural killer</td>
</tr>
<tr>
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<td>cytotoxic T lymphocytes</td>
</tr>
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<td>mouse embryonic fibroblast</td>
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<td>vaccinia virus</td>
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<td>PFU</td>
<td>plaque forming unit</td>
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<td>AGM₁</td>
<td>asialo GM₁</td>
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Introduction

Natural killer cells become activated (reviewed in 1) and proliferate (2,3) during the early stages of virus infections, and it has been hypothesized that they may play a role in natural resistance to viruses (1). Although there have been numerous demonstrations of NK cell-mediated lysis of virus-infected cells in vitro (reviewed in 4), there has been no definitive demonstration of an antiviral role for NK cells in vivo. Suggestive data in support of this hypothesis have all been subject to other interpretations. The main approaches have been to correlate susceptibility to virus infection with NK activity which was either determined genetically or manipulated experimentally.

In this regard, perhaps the best evidence has been gathered for murine cytomegalovirus (MCMV)³ infection. Shellam and coworkers have shown that genetic resistance of mice to MCMV correlates with their NK cell activity (5,6), that homozygous beige mice, which have a defect in NK cell-mediated cytotoxicity, are highly susceptible to MCMV, and that resistance along with NK cell activity is restored to beige mice by transfers of bone marrow cells from normal mice (6). Newborn mice, which have low NK cell activity, are highly sensitive to MCMV (7), as are adult mice whose NK cell activity has been depleted by nonspecific immunosuppression (8,9). Recently, we have shown that antiserum to asialo GM₁, a reagent which rather selectively eliminates NK cell activity in mice, greatly enhances MCMV growth and pathogenesis (10,11). Biological response modifiers, which activate NK cells in vivo in the absence of IFN, render mice more resistant to MCMV; this resistance is abrogated by antiserum to asialo GM₁ (12).
The above experiments provide a strong argument for a role of NK cells in resistance to MCMV, but they are not definitive. For example, beige mice have a biochemical defect which affects functions other than NK cells (13), and asialo GM₁ is a common molecule found on several cell types (14,15). To more definitively assess the role of NK cells in virus infections, we have developed cellular adoptive transfer methods to identify the effector cells mediating natural resistance. These methods are used to manipulate infections with MCMV and with lymphocytic choriomeningitis virus (LCMV), which appears to resist NK cells. LCMV synthesis is normal in beige mice (16) and in mice treated with anti-asialo GM₁ (10). We report here that transfer only of those populations of cells containing NK cell activity can protect suckling mice against MCMV but not LCMV. Further, adoptive transfer of cloned NK but not T cells provide resistance to MCMV but not to LCMV. These data provide compelling evidence in favor of a role for NK cells in at least one (MCMV) virus infection.
Materials and Methods

Animals. C57BL/6 and BALB/c mice were purchased from the Jackson Laboratories, Bar Harbor, ME, then bred in our own facility. SWR/J athymic nude mice were a gift from Dr. Aldo Rossini, University of Massachusetts Medical Center. Donor mice in adoptive transfer studies were 4-8 wks old, unless otherwise noted.

Cells. YAC-1 cells were derived from a Moloney leukemia virus-induced lymphoma from an A/Sn mouse and were maintained in RPMI-1640 medium (GIBCO, Grand Island, NY) supplemented with antibiotics, glutamine, and 10% heat-inactivated fetal bovine serum (FBS) (M.A. Bioproducts, Walkersville, MD). Mouse embryo fibroblasts (MEF) were obtained as described (17) from C57BL/6 embryos. Vero cells are a continuous monkey kidney cell line. L-929 is a continuous liver cell line derived from C3H mice. These cells were maintained in minimal essential medium (MEM) (GIBCO) with the same additives as listed above. Baby hamster kidney (BHK) 21/13S cells were maintained in Dulbecco's modified Eagle medium (GIBCO) supplemented with 10% tryptose phosphate broth and the same additives as listed above.

Viruses. The Smith strain of MCMV was obtained from Dr. John Nedrud, Case Western Reserve University School of Medicine, Cleveland, OH (18). Salivary glands from BALB/c mice inoculated 2 to 3 wk previously with $10^4$ plaque forming units (PFU) of MCMV were homogenized in a 10% suspension and cleared by centrifugation. Aliquots were stored at -70°C in 10% dimethylsulfoxide. The LCMV used in these studies was the
Armstrong strain, which was grown in BHK cells (19). The Indiana strain of vesicular stomatitis virus (VSV) was used in the IFN assays.

**Cytotoxicity Assay.** Assay medium was RPMI-1640 supplemented with 0.1 M N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES) (Sigma Chemical Co., St. Louis, MO), 10% FBS, glutamine, and antibiotics. The assay was performed as described (19). Briefly, target cells labeled with 100 uCi sodium chromate (New England Nuclear, Boston, MA) for 1 hr at 37°C were washed and mixed with various numbers of effector cells in round-bottomed microtiter wells at $10^4$ target cells/well. For spontaneous release determinations, medium was added to the wells, and for maximum release determinations, 1% Nonidet P-40 was added. Plates were incubated for 4 to 16 hr at 37°C in a humidified atmosphere of 5% CO$_2$ and 95% air. At the end of the incubation, plates were centrifuged at 200 X g for 5 min, and 0.1 ml of the supernatant was collected and counted for radioactivity in a Beckman Gamma 5500 counter (Beckman Instruments, Palo Alto, CA). Data are expressed as percent specific release:

$$100 \times \frac{\text{cpm experimental} - \text{cpm spontaneous}}{\text{cpm maximum} - \text{cpm spontaneous}}$$

Spontaneous release of radioactivity was between 10 and 20%. Standard deviations of quadruplicate replica samples were less than 10% of the mean and were not listed in the tables.
Virus titration. Spleen and liver virus titers were determined by using a 10% homogenate of tissue taken from individual mice. The number of PFU was determined by plaque assay using MEF and Vero cells for MCMV and LCMV, respectively. Virus titers are reported per spleen and per gram of liver. Results are expressed as the geometric mean titer, i.e., the arithmetic averages of the logs of four separate animals titrated for virus individually ± the standard error of the mean (SEM). The P values represent the significance of the differences of the means between the designated sample and the appropriate control, and were calculated using Student's t-test.

Antisera. Rabbit antiserum to asialo GM₄ was purchased from Wako Chemicals, USA, Inc., Dallas, TX. This antiserum has previously been shown to selectively deplete NK cell activity in vivo and in vitro (14,15,20). To deplete NK cell activity in vivo, anti-asialo GM₄ was diluted 1/10 in RPMI medium and given i.v. in a volume of 0.2 ml, 4-6 hr before challenge with virus. In adoptive transfer experiments, donor spleen cells from control mice treated with anti-asialo GM₄ were used 18-24 hrs after antibody treatment. Monoclonal anti-thy 1.2 antibody was provided by Dr. Edward Clark, Genetic Systems Corp., Seattle, WA and used at a final dilution of 1:900. Monoclonal anti-Ly 5.1 (clone M1/89.18) and anti-Ia (clone M5/114) antibodies, gifts from Dr. Eric Martz, University of Massachusetts, Amherst, MA (21) were used at final dilution of 1:40 and 1:100, respectively. The antiserum to NK 1.2 was a gift from Dr. Robert Burton (22) and was used at a final dilution of 1:40. For in vitro treatment of spleen cells, 2.5 - 4.0 x 10⁸ spleen leukocytes were suspended in 2.5 ml RPMI containing antibody and
incubated for 20-30 min at room temperature with occasional agitation. Two hundred and fifty ul of guinea pig serum (a source of C') was then added and the incubation was continued at 37°C for an additional 45 min. The cells were washed twice and resuspended in RPMI for use in adoptive transfer and cytotoxicity assays. The cell numbers transferred refer to the number of viable cells before the antibody and C' treatment.

**Cloned NK cells.** NK186B10, a clone of IL-2-dependent NK cells derived from C57BL/6 mice, was maintained as described (23), with a few modifications. Growth medium was RPMI-1640 supplemented with 2mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM MEM non-essential amino acids (GIBCO), 5 x 10^{-5} M 2-mercaptoethanol, 50 U/ml penicillin and streptomycin, 100 ug/ml gentamycin, 5% FBS, and 24% supernatant derived from concanavalin A-stimulated mouse spleen cells as a source of IL-2. For use in experiments, cells growing in 25 or 75 cm² flasks were washed and adherent cells were removed with trypsin-EDTA (GIBCO) and diluted 1:4 in growth medium. This suspension was then incubated at 37°C for 1 hr in a conical test tube, with occasional agitation. Cells were then counted, pelleted at 200 x g for 5 min, and resuspended in RPMI for use in cytotoxicity assays and adoptive transfers. As a control, a C57BL/6-derived cytotoxic T line (CTLL-2 clone 15 H) (24), routinely used as indicator cells in IL-2 bioassays, were also used.

**Adoptive transfers.** Recipients were C57BL/6 suckling mice 3-5 days of age. Before use in experiments, mice from several litters were pooled and randomly assigned to lactating mothers. Groups of 4-9 mice were given an injection of cloned NK cells or mouse spleen cells treated in various ways. The volume was 0.1 ml delivered i.p. using a 1 ml
syringe and a 30 gauge needle. The next day, mice were challenged with $6 \times 10^3$ PFU of MCMV or $8 \times 10^3$ PFU of LCMV i.p. in a volume of 0.1 ml. Mice were either monitored daily for survival, or sacrificed 3 days later and their spleens removed for virus titration (see Fig. 1A). In some experiments using the cloned NK cells (23) four-week old C57BL/6 mice were given four weekly doses of 200 rads of $\gamma$ radiation from a $^{60}$Co cobalt source. Five weeks after the last dose, the mice were given 10 ul anti-asialo GM$_1$ i.v., followed 2 days later by $4 \times 10^6$ cloned NK cells, half given i.p., the other half given i.v. Six days later, i.p. and i.v. injections were again administered, each consisting of $10^6$ cloned NK cells. Ten days after the last injection, groups of 5 mice were challenged with an i.p. injection of $10^4$ PFU of MCMV. Controls consisted of irradiated, anti-asialo GM$_1$-treated mice not receiving injections of NK cells. Three days later, the mice were sacrificed, and their spleens and livers were titrated for virus (see Fig. 1B).

**Interferon assay.** Blood was collected in heparinized Natelson tubes from the retro-orbital sinus of mice anaesthetized with ether, and plasma was obtained by centrifugation. Plasma were titrated by two-fold serial dilution in a 96-well, flat-bottomed microtiter plate to which L-929 cells were added at $3 \times 10^4$ cells/well. Eighteen to 24 hr later, the wells were challenged with 100 TCID$_{50}$ units of VSV. IFN titers were expressed as the log$_2$ of the highest reciprocal dilution resulting is a 50% reduction in cytopathic effect. Results are expressed as the geometric mean titers of separate animals titrated for IFN individually + SEM.
**Centrifugal elutriation.** Size separation of cells was accomplished using a Beckman JE-6B centrifuge (2). About $1 \times 10^9$ spleen leukocytes were treated with deoxyribonuclease (Sigma) to prevent clumping. These cells were then loaded into the rotor which was spinning at 3200 rpm at 5°C. The rate of flow of elution medium (Hank's balanced salt solution, 1.5% calf serum) was 15, 22, 28, and 46 ml/min and corresponded to fractions 1 through 4, respectively. Cell recovery was about 65%. Fraction 2 was used as a source of purified lymphocytes.

**Enrichment of NK cells.** Spleen leukocytes ($1.6 \times 10^9$) were passed through nylon wool columns and then further separated on discontinuous Percoll gradients according to the method of Kumagai et al. (25) with some modifications. One part 10X phosphate buffered saline (PBS) and one part FBS were added to 8 parts Percoll to obtain 80% Percoll. This was further diluted using RPMI-1640 containing 10% FBS. Six to 7 X $10^7$ cells were suspended in 3 ml 64% Percoll and pipetted into a 15 ml conical test tube. Three ml of 59%, 50%, and 37% Percoll were sequentially layered on top of the cell suspension. Centrifugation at 300 x g at 20°C was 45 min in duration. Cells at the very top of the gradient were discarded. Cells floating atop the 59 and 50% Percoll bands were pooled, and those atop the 63% band were pooled with those in the pellet. The latter cells were found to be devoid of NK cell activity, and were used in adoptive transfer experiments. The cells from the 59 and 50% bands were pooled and further purified on a second Percoll gradient. Six x $10^7$ of these cells were suspended in 6 ml 59.5% Percoll and 3 ml of 55.0 and 37.0% Percoll were sequentially
layered on top of the cell suspension. Centrifugation was carried out as before, and cells floating atop the 55.0% Percoll band were washed and used in adoptive transfer studies, as they were found to be enriched for NK cell activity.
Results

Protection against lethal MCMV by adoptive transfer of adult spleen leukocytes or lymphocytes. Suckling mice have low NK cell activity and are much more susceptible to MCMV infection than adult mice (7), and Selgrade and Osborn have shown that adult spleen leukocytes could protect suckling mice against lethal MCMV infection (17). Figure 2A shows that adoptive transfer of $5 \times 10^7$ leukocytes from 4-8 wk old mice significantly prolonged the survival of mice lethally infected with MCMV, but that transfer of $1.7 \times 10^7$ or fewer leukocytes did not. Figure 2B shows that $5 \times 10^7$ spleen leukocytes from 5 wk old donors were significantly more effective than those from 17 day old donors in prolonging the lives of MCMV-infected suckling mice. To further define which population of leukocytes was providing protection, we obtained purified spleen lymphocytes by performing centrifugal elutriation on unseparated spleen cells. Fraction 2 was routinely found to contain about 98% lymphocytes and < 1% of the cells were capable of phagocytizing yeast particles, indicating minimal contamination with macrophages. Figure 2C shows that these purified lymphocytes were more capable than unfractionated leukocytes in prolonging survival. These experiments demonstrate that protection can be mediated by a population of spleen lymphocytes present in adult but not 17 day old mice.

T cell depletion does not affect protective capacity. Spleen leukocytes were depleted of T cells by treatment with monoclonal anti-thy 1.2 and C' and then adoptively transferred into recipient suckling
mice. This treatment kills about 35% of the cells, eliminates LCMV-specific cytotoxic T cell activity of sensitized spleen cells, but has little effect on NK cell activity (19). Figure 2 D shows that T cell-depleted spleen leukocytes were just as protective as those treated with C' only, leading us to conclude that protection occurs in the absence of adoptively transferred T cells. Another line of evidence indicating that protection can occur without T cells is shown in Figure 3. Athymic nude mice were either left untreated or selectively depleted of NK cell activity by injection with anti-asialo GM₁ antibody. Four to six hours later, the mice were challenged with MCMV. The data show that MCMV-infected NK cell-depleted mice had 5-6 times more liver virus and 2-3 times more spleen virus than control MCMV-infected mice. This provides evidence that T cells are not required for the anti-asialo GM₁ antiserum-mediated exacerbation of MCMV infection. Depletion of NK cells results in loss of protection. Spleen leukocytes from adult mice either untreated or NK cell-depleted by injection with anti-asialo GM₁ 18-24 hr earlier were adoptively transferred into suckling mice. This treatment reduced NK cell activity from 21 to 2.7%. Figure 2 E shows that the cells from mice depleted of NK cell activity by anti-asialo GM₁ failed to prevent the death of mice infected with MCMV, whereas control leukocytes totally prevented death. Treatment of leukocytes with monoclonal anti-Ly 5 antibody and C' reduced NK cell activity from 20 to 8.6% and eliminated the protection, but treatment with monoclonal anti-Ia antibody and C', which killed 40% of
spleen leukocytes, but did not deplete NK cell activity, had no effect on the ability of spleen cells to protect (Fig. 2 F). This shows that leukocytes depleted of B cells and other Ia-bearing cells can still protect against lethal infection.

Reduction of spleen virus titers by adoptive transfer of NK-enriched Leukocytes. Since previous studies showed that increased survival correlates with lower spleen MCMV titers (11), we titrated the spleens of MCMV-infected suckling mice 3 days postinfection. The data in Table 1 A, B, C, D, show that those mice receiving control adult spleen leukocytes had significantly lower spleen MCMV titers than those receiving no cells. Transfer of nylon wool-passed cells also reduced MCMV titers in recipients, confirming the data in Figure 2 showing that cells depleted of B cells and macrophages can protect (Table 1 A). Nylon wool-passed leukocytes were next subfractionated in Percoll gradients to enrich for or to deplete NK cells (Table 1 B). The experiment depicted in Figure 2 A suggested that $1.7 \times 10^7$ control spleen cells did not enhance survival, but that three times as many of these cells did. Table 1 B shows that $1.7 \times 10^7$ control spleen cells resulted in only a 3-5 fold reduction in MCMV titers in the recipient spleens, but that the same number of cells enriched 3-fold for NK cell activity in low density fractions after Percoll purification resulted in about a $1.5 \log_{10}$ (50-fold) decrease in MCMV titers. Mice receiving an equal number of Percoll-separated high-density cells devoid of NK cell activity were not at all protected. Thus, nylon wool passed, Percoll gradient fractionated spleen cells enriched for NK cell activity
have an enhanced capacity to reduce virus titers as compared to control spleen leukocytes or those devoid of NK cell activity.

**Failure of NK-depleted leukocytes to reduce virus titers.** Immunochemical depletion of NK cell activity from the donor spleen before adoptive transfer drastically reduced the ability of the spleen cells to inhibit MCMV synthesis in recipients. Transfer of adult spleen cells treated with anti-NK 1.2 and C' (Table 1 C) or spleen cells from anti-asialo GM1-treated mice (Table 1 D) resulted in recipient spleen MCMV titers which were nearly a log_{10} higher than those of recipients receiving control adult spleen cells.

**Cloned NK cells provide protection against MCMV.** To further strengthen the evidence that NK cells are mediating antiviral effects *in vivo*, we used the cloned IL-2-dependent NK cell line NK1B6B10 as donor cells in adoptive transfer experiments. The data in Table 1 D, E show that these cells were extremely effective in reducing MCMV titers, as mice receiving the cloned NK cells had about 500-fold less MCMV in their spleens than did mice receiving either no cells or an IL-2-dependent T cell clone (CTLL-2 clone 15 H) (24) also derived from C57BL/6 mice. On a cell-to-cell basis the NK clone was greater than 100-times more effective than adult leukocytes, as 5 \times 10^5 of the cloned cells were far more effective at reducing MCMV titers than were 5 \times 10^7 leukocytes. These experiments show that an NK cell clone by itself can limit MCMV replication, and that an irrelevant T cell clone cannot. Cloned NK cells were also capable of enhancing the survival of MCMV-infected mice, as 75% of mice receiving only 3 \times 10^5 NK cells survived, as compared to 0% of control mice (Fig. 4).
To test whether the NK clone could protect adult mice from MCMV, 6-week-old mice were irradiated four times with 200 Rads at weekly intervals to deplete NK cell activity and then given injections of the cloned NK cells. The reconstituted recipients had elevated spleen NK activity and reduced virus titers in their spleens and livers (Fig. 5). IFN levels were slightly higher in the unreconstituted mice, which synthesized more virus. The experiments provide evidence that NK cells can mediate antiviral effects in adult as well as suckling mice.

Adoptive transfer of NK cells has no effect on LCMV titers. Previous evidence (10,16) has suggested that NK cells do not play a role in limiting LCMV synthesis during acute or persistent infection. To test this hypothesis using our adoptive transfer system, we transferred either $5 \times 10^7$ adult spleen cells or $5 \times 10^5$ cloned NK cells into suckling mice and then challenged them the next day with LCMV. Table 2A, B shows that the transfer had no effect on LCMV synthesis in the spleens of recipient mice. Thus, adoptive transfer of NK cells markedly inhibited MCMV synthesis but had no effect on LCMV synthesis.
Discussion

The results presented in this paper provide compelling evidence that NK cells play a major role in limiting MCMV synthesis and MCMV-induced mortality. Table 3 summarizes this evidence and collectively shows that the phenotype of the protective cell population is that of a nylon wool non-adherent, asialo GM1⁺, NK 1.2⁺, Ly 5⁺, thy-1⁻, Ia⁻, low-density lymphocyte. This describes an NK cell (reviewed in 26). In each case, the presence or absence of NK cell activity in the adoptively transferred population correlated with resistance and sensitivity to MCMV, respectively.

Because data for survival curves must be accumulated over a period of 2-3 weeks, it is possible that over that period of time subpopulations of spleen cells could differentiate and subsequently provide resistance, making the results difficult to interpret. However, in many experiments, suckling mice receiving NK cells were larger and weighed more than mice not receiving NK cells, even three days postinfection (data not shown). Previous studies with anti-asialo GM₁ suggested that NK cells could mediate their antiviral effects up to 3 days postinfection, but not at 6-9 days (11). The present experiments involving titration of MCMV in the spleen 3 days postinfection confirm this result and indicate that the results are not due to differentiation and sensitization of T cells over the long time period. T cells are reported to mediate antiviral effects 6-30 days postinfection (27). Further arguments against a role for T cells early in infection
include the fact that NK depletion lowers the resistance of athymic nude mice to MCMV infection (Fig. 3) and the lack of effect of T cell depletion by antiserum to thy 1.2 plus C' on the antiviral action of transferred spleen cells (Fig. 2 D). Whereas some NK cells express some thy 1 antigen (26), the concentrations of antiserum and C' used here deplete CTL activity but have little effect on endogenous NK cell activity (19).

Selgrade and Osborn (17) concluded that either unstimulated macrophage-depleted spleen leukocytes or thioglycollate-induced peritoneal macrophages could enhance survival of MCMV-infected suckling mice. However, the nature of the spleen leukocyte was not identified. It was not shown whether unstimulated macrophages could provide resistance to MCMV, as the macrophages used in those studies were thioglycollate-induced. Our studies were not designed to answer this question, but nevertheless show that spleen cells depleted of macrophages by nylon wool passage or size separation retained the capability to provide protection. Thus, adoptive transfer of macrophages is not essential to transfer of resistance. Antibodies which leave macrophage function intact, such as anti-asialo GM1, anti-Ly 5, and anti-NK 1.2, eliminate both NK cell activity and protection against MCMV.

There has been little evidence to implicate neutrophils in resistance to MCMV. Our adoptive transfer experiments show that populations of spleen lymphocytes with > 98% purity are quite capable of mediating resistance. Neutralizing antibody can be detected as early as 3 days postinfection of adult mice, and thus could possibly mediate protection
(28), but our experiments show that depletion of B cells by treatment with anti-Ia and C' or by nylon wool passage did not remove the protective capacity of spleen cells, indicating that adoptive transfer of B cells was not required for protection.

The survival curves indicated that $5 \times 10^7$ adult spleen cells could prolong survival of MCMV-infected mice, but that 3-fold fewer cells could not. If the protection were mediated by NK cells, then 3-fold fewer spleen cells with three times as much NK cell activity should protect. This prediction was supported by our data, as $1.7 \times 10^7$ unfractionated spleen cells or the same number of NK cell-deficient spleen cells did not fully protect, while $1.7 \times 10^7$ spleen cells enriched 3-fold for NK cell activity significantly reduced MCMV titers.

Previous work using an IL-2-dependent cloned NK cell line showed that these cells were capable of mediating resistance to tumor implants and bone marrow transplants (29). These cells have the phenotype asialo GM$_1^+$, NK 1.2$^+$, thy-1$^+$, Ly $1^-$, Ly $2^-$ and are capable of mediating in vitro lysis against NK-sensitive YAC-1 tumor cells (23). Our results show that these cells are capable of providing protection against MCMV, but not LCMV. In contrast, a cloned T cell line also derived from C57BL/6 mice provided no protection, indicating that cloned lymphoid cells in general do not necessarily protect. The data obtained with cloned NK cells also indicate that no other adoptively transferred cell population was needed for protection; NK cells either directly mediated resistance or were solely responsible for triggering recipient defense mechanisms.
We conclude from our results that NK cells can provide protection against at least one (MCMV) but not all (LCMV) virus infections. There are other data consistent with the concept that NK cells play a role in murine infections with herpes simplex virus (30), mouse hepatitis virus (10,31), Friend leukemia virus (32), and vaccinia virus (10). There also is evidence consistent with the hypothesis that NK cells may inhibit outgrowth of tumor cells persistently infected with measles or vesicular stomatitis virus (33). Evidence against a role for NK cells has been provided for LCMV (10,16) and Sindbis virus (34). Why NK cells should play a role in some but not all virus infections is not known, but the accompanying paper shows that the NK cell system may have an ability to selectively lyse cells infected with MCMV, an NK-sensitive virus, but not LCMV, an NK-resistant virus.
Summary

Adoptive transfer studies were carried out to determine the role of NK cells in resistance to murine cytomegalovirus (MCMV) and lymphocytic choriomeningitis virus (LCMV). Leukocytes from adult mice were transferred into suckling mice 1 day prior to infection with virus. Resistance was measured by enhancement of survival and reduction of virus synthesis in the spleens of recipient mice. The phenotype of the cell population capable of mediating resistance to MCMV was that of a nylon wool non-adherent, asialo GM$_1^+$, NK 1.2$^+$, Ly 5$^+$, thy-1$^-$, Ia$^-$, low-density lymphocyte; this is the phenotype of an NK cell. Cloned NK cells but not cloned T cells provided resistance to MCMV in suckling mice. Cloned NK cells also provided resistance to MCMV in irradiated adult mice, and antibody to asialo GM$_1$, which depletes NK cell activity in vivo, enhanced the synthesis of MCMV in athymic nude mice. Neither adult leukocytes nor cloned NK cells influenced LCMV synthesis in suckling mice. We conclude that a general property of NK cells may be to provide natural resistance to virus infections and that NK cells could protect mice from MCMV but not from LCMV.
References


34. Hirsch, R.L. 1981. Natural killer cells appear to play no role in the recovery of mice from Sindbis virus infection. Immunology. 43: 81.
Table 1

Reduction of MCMV titers by adoptive transfer of cell populations containing NK cell activity\(^a\)

<table>
<thead>
<tr>
<th>Exp.</th>
<th>E:T by donor cells</th>
<th>cells/treatment</th>
<th>% NK lysis(^b)</th>
<th>log(_{10}) PFU MCMV/spleen</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>medium</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td></td>
<td>5 ( \times ) 10(^7) control spleen</td>
<td>N.D.</td>
<td>4.1 ± 0.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5 ( \times ) 10(^7) nylon wool-passed spleen</td>
<td>2.5 ± 0.2(^c)</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>6</td>
<td>1.7 ( \times ) 10(^7) NK-depleted</td>
<td>3.1 ± 0.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>39</td>
<td>1.7 ( \times ) 10(^7) NK-enriched</td>
<td>1.5 ± 0.2(^d)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>1.7 ( \times ) 10(^7) control spleen</td>
<td>2.4 ± 0.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>5.0 ( \times ) 10(^7) control spleen</td>
<td>1.4 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>25</td>
<td>5 ( \times ) 10(^7) control spleen</td>
<td>medium</td>
<td>4.1 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>29</td>
<td>5 ( \times ) 10(^7) control spleen + C(^\prime)</td>
<td>2.7 ± 0.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>5 ( \times ) 10(^7) control spleen + anti-NK + C(^\prime)</td>
<td>3.7 ± 0.1(^e)</td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>50</td>
<td>5 ( \times ) 10(^7) control spleen</td>
<td>3.2 ± 0.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>5 ( \times ) 10(^7) anti-AGM(_1) spleen</td>
<td>4.1 ± 0.1(^f)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>N.D.</td>
<td>5 ( \times ) 10(^7) cloned NK cells</td>
<td>1.9 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>20</td>
<td>5 ( \times ) 10(^5) cloned NK cells</td>
<td>medium</td>
<td>4.5 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>1.2</td>
<td>5 ( \times ) 10(^5) cloned T cells</td>
<td>1.8 ± 0.2(^g)</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) NK cell activity was determined by the percentage of NK lysis as measured by in vitro 51Cr release assays.

\(^b\) % NK lysis was calculated as (cPMN + 100)/(cPMN + TPMN).

\(^c\) Means \(±\) SEM, \(n = 6\), compared to MCMV/spleen mean of 1.9 ± 0.1.

\(^d\) Means \(±\) SEM, \(n = 6\), compared to MCMV/spleen mean of 1.9 ± 0.1.

\(^e\) Means \(±\) SEM, \(n = 6\), compared to MCMV/spleen mean of 1.9 ± 0.1.

\(^f\) Means \(±\) SEM, \(n = 6\), compared to MCMV/spleen mean of 1.9 ± 0.1.

\(^g\) Means \(±\) SEM, \(n = 6\), compared to MCMV/spleen mean of 1.9 ± 0.1.
Table 1 - Continued

a Groups of 4 suckling mice were given i.p. injections of cells treated as indicated above, or RPMI-1640 medium. The next day, they were challenged with $6 \times 10^3$ PFU of MCMV. Three days later the mice were sacrificed and their spleens were titrated for MCMV.

b % NK lysis refers to % specific release against YAC-1 targets at a given effector:target (E:T) ratio, as described in Materials and Methods.

$^{c}p < .001$ as compared to medium.

$^{d}p < .01$ as compared to $1.7 \times 10^7$ control spleen.

$^{e}p < .01$ as compared to control spleen + C'.

$^{f}p < .01$ as compared to control spleen.

$^{g}p < .001$ as compared to cloned T cells.
Table 2

Adoptive transfer of cell populations containing NK cell activity has no effect on LCMV titers.

<table>
<thead>
<tr>
<th>Exp</th>
<th>Cells/treatment</th>
<th>log_{10} pfu</th>
<th>LCMV/spleen</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>medium</td>
<td>4.7 ± 0.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5 x 10^7 control spleen</td>
<td>4.4 ± 0.1^b</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>medium</td>
<td>4.7 ± 0.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5 x 10^5 cloned NK cells</td>
<td>4.7 ± 0.2^b</td>
<td></td>
</tr>
</tbody>
</table>

^a Groups of 4 suckling mice were given i.p. injections of cells treated as indicated above, or RPMI medium. The next day, they were challenged with 8 x 10^3 of PFU LCMV. Three days later, the mice were sacrificed and their spleens were titrated for LCMV.

^b Not significantly different from medium.
Table 3
Summary of adoptive transfer experiments

<table>
<thead>
<tr>
<th>Cells/treatment</th>
<th>NK cell activity</th>
<th>antiviral effect against MCMV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control adult spleen leukocytes</td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td>Control adult spleen lymphocytes</td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td>17-day-old spleen leukocytes</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>Control spleen + anti-thy 1.2 + C'</td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td>Control spleen + anti-Ia + C'</td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td>Control spleen + anti-Ly 5 + C'</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>Spleen leukocytes from anti-asialo GM-treated mice</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>Control spleen + anti-NK 1.2 + C'</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>Cloned T cells</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>Cloned NK cells</td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td>Low-density spleen cells</td>
<td>yes</td>
<td>yes</td>
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<tr>
<td>High-density spleen cells</td>
<td>no</td>
<td>no</td>
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</tbody>
</table>
Figure Legend

Figure 1. Adoptive transfer protocols. A. Cloned NK cells or spleen leukocytes from adult C57BL/6 mice 6-8 week old were fractionated and given i.p. to suckling mice 3-5 days old. The next day, MCMV or LCMV was given i.p. The mice were either monitored for survival for 20 days, or sacrificed 3 days postinfection and their spleens titrated for virus. B. Adult C57BL/6 mice were given four weekly doses of radiation and then given cloned NK cells i.p. and i.v. The mice were then challenged with $10^4$ PFU of MCMV i.p. and were sacrificed 3 days later.

Figure 2. Effect of adoptive transfer of various spleen cell populations on survival of MCMV-infected suckling mice. Suckling mice 3-5 days old were given $5 \times 10^7$ spleen cells (unless otherwise indicated) or medium i.p. followed 24 hr later by $6 \times 10^3$ PFU of MCMV i.p. Mice were monitored for survival for 20 days. A. Various numbers of adult spleen leukocytes were transferred. $N = 5$ mice/group. B. Spleen leukocytes from suckling and weanling mice were used as donor cells. $N = 5-6$ mice/group. C. Spleen leukocytes or size-separated lymphocytes were used as donor cells. $N = 6-7$ mice/group. D. Spleen leukocytes treated with C or anti-thy 1.2 and C were used as donor cells. $N = 7$ mice/group. E. Spleen leukocytes from control or anti-asialo GM$_1$-treated mice were used as donor cells. $N = 7-9$ mice/group. F. Spleen leukocytes treated with C', anti-Ly 5 and C', or anti-Ia and C' were used as donor cells. $N = 6$ mice/group.
**Figure 3.** NK cell depletion lowers resistance to MCMV in nude mice. Groups of four SWR/J athymic nude mice were left untreated or given 20 μl anti-asialo GM₁ i.v. Four to 6 hr later, these mice were challenged with 5 X 10³ PFU of MCMV i.p. Three days later, the mice were sacrificed and their organs were titrated for MCMV, their spleens were assayed for NK cell activity, and their plasma was titrated for IFN.

**Figure 4.** Cloned NK cells enhance survival of MCMV-infected suckling mice. Cloned NK cells were adoptively transferred into recipient suckling mice and then challenged 24 hr later with 6 X 10³ PFU of MCMV. Control mice received no cells. Mice were observed 20 days for mortality. N = 8 mice/group.

**Figure 5.** Cloned NK cells inhibit MCMV replication in adult mice. Adult C57BL/6 mice were irradiated and left untreated or injected with cloned NK cells as described in Materials and Methods. These mice were then challenged with 10⁴ PFU of MCMV i.p. and sacrificed three days later. Spleens and livers were titrated for MCMV, spleens were assayed for NK cell activity, and plasma was titrated for IFN.
Figure 1

A

Fractionate

1 Day

MCMV

Survival Curve

3 Days

Titrate Organs

5 days old

B

200 R

NK Clone

1 Week

NK Clone

1 Week

MCMV

Titrate Organs

Figure 1
Figure 2
Figure 3
Figure 4

PERCENT SURVIVAL

DAYS POSTINFECTION

3 \times 10^5 NK cells

no cells
Figure 5
Susceptibility of Virus-Infected Targets to Natural Killer
Cell-Mediated Lysis In Vitro Correlates With
Natural Killer Cell-Mediated Antiviral Effects In Vivo*

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Abbreviations:

<table>
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<tr>
<th>Abbreviation</th>
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<tr>
<td>LCMV</td>
<td>lymphocytic choriomeningitis virus</td>
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<tr>
<td>MCMV</td>
<td>murine cytomegalovirus</td>
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<tr>
<td>VV</td>
<td>vaccinia virus</td>
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<tr>
<td>MOI</td>
<td>multiplicity of infection</td>
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<td>MEF</td>
<td>mouse embryo fibroblast</td>
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<td>NK</td>
<td>natural killer</td>
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<td>IFN</td>
<td>interferon</td>
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<td>CPE</td>
<td>cytopathic effect</td>
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<td>SEM</td>
<td>standard error of the mean</td>
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<td>MHV</td>
<td>mouse hepatitis virus</td>
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Introduction

Since the augmentation of natural killer (NK) cell activity during acute viral infection was first described (1-4), circumstantial evidence that NK cells may play a role in the host defense against virus infections has been accumulating (5-8). Much of this evidence has been obtained using acute murine cytomegalovirus (MCMV) infection as a model. Mice with low NK cell activity caused by lack of age (9), genetic predisposition (10), immunosuppressive drug treatment (11), or the beige mutation (8) are more susceptible to MCMV infection. Selective depletion of NK cell activity by injection with antibody to asialo GM₁ results in increased virus synthesis and severe pathological changes in MCMV-infected mice (12,13). In contrast to the results with MCMV, all the available evidence suggests that NK cells do not protect mice against lymphocytic choriomeningitis virus (LCMV) infection (12,14,15). We have recently shown that nylon wool non-adherent, asialo GM₁⁺, NK 1.2⁺, Ly 5⁺, thy-1⁻, Ia⁺, low-density lymphocytes (this is the phenotype of NK cells) when adoptively transferred to NK-cell deficient suckling mice result in enhanced survival and reduced virus titers in MCMV-infected but not LCMV-infected recipients. Further, IL-2-dependent cloned NK cells also provided resistance to MCMV, but not LCMV.

Both LCMV and MCMV induce the activation of NK cells in vivo (10,16), but it is not known why NK cells provide resistance to MCMV but not LCMV. Some evidence (17) suggests that MCMV can induce NK
cells with characteristics different from those induced by other viruses. It is thus possible that MCMV is capable of inducing a type of specialized antiviral NK cell whereas LCMV is not.

A possible mechanism whereby NK cells could mediate antiviral effects is by preferential lysis of virus-infected cells over uninfected cells. Endogenous spleen cells containing NK cell activity lyse MCMV-infected cells more efficiently than uninfected cells (18,19), but this is not known to occur in the LCMV system (16). Activated NK cells lyse LCMV-infected and uninfected cells to the same degree (16,20), but MCMV-infected cells are less sensitive than uninfected cells to lysis by effectors resembling activated NK cells (10). This decreased sensitivity of virus-infected targets to activated NK cells has also been observed with several other virus-cell systems (20).

Trinchieri and coworkers (21) made the observation that human target cells could be protected from NK cell-mediated lysis by interferon (IFN) pretreatment, but not if they were infected with vaccinia (VV) or influenza viruses. Further, they demonstrated that target cells treated with inhibitors of protein or RNA synthesis could not be protected by IFN (21), and this was confirmed in the murine system (22). Because MCMV shares with influenza and vaccinia viruses the ability to induce a cytopathic effect (CPE), and because LCMV is relatively noncytopathic (23), we tested the hypothesis that LCMV- but not MCMV- or VV-infected cells could be protected from NK cell-mediated lysis by IFN.
In this paper, we characterize the endogenous and MCMV-activated effectors capable of lysing MCMV-infected targets as NK cells, and we show that IFN pretreatment almost totally protects uninfected and LCMV-infected cells, while leaving MCMV- or VV-infected cells susceptible to lysis by activated NK cells. We also show that selective depletion of NK cell activity in vivo results in increased titers of spleen MCMV but not LCMV in mice simultaneously infected with both viruses. These data could explain why NK cells may play a role in resistance to MCMV and VV, but not LCMV.
Materials and Methods

Animals. C57BL/6 and BALB/c byJ mice were purchased from the Jackson Laboratory, Bar Harbor, ME. C3H/St mice were purchased from West Seneca Laboratories, West Seneca, NY. Mice of either sex 6 to 10 wk old were used in these experiments.

Cells. YAC-1 cells were derived from a Moloney leukemia virus-induced lymphoma in A/Sn mice and were maintained in RPMI 1640 medium (GIBCO, Grand Island, NY) supplemented with antibiotics, glutamine, and 10% heat-inactivated fetal bovine serum (M.A. Bioproducts, Walkersville, MD). L-929 is a continuous cell line from C3H mice. Mouse embryo fibroblasts (MEF) were obtained as described (24) from C57BL/6 embryos. Vero cells are a continuous monkey kidney cell line. These cells were maintained in minimal essential medium (GIBCO) with the same additives as listed above. Baby hamster kidney (BHK 21/13S) cells were maintained in Dulbecco's modified Eagle medium (GIBCO) supplemented with 10% tryptose phosphate broth and the same additives as listed above.

Viruses. The Smith strain of MCMV was obtained from Dr. John Nedrud, Case Western Reserve University School of Medicine, Cleveland, OH (25). Salivary glands from BALB/c mice inoculated 2 to 3 wk previously with $10^4$ PFU of MCMV were homogenized in a 10% suspension and cleared by centrifugation. Aliquots were stored at -70° C in 10% dimethylsulfoxide. For infection of target cells, salivary gland virus was passed in MEF 3 times, and aliquots were stored at -70° C without dimethylsulfoxide. The LCMV used in these studies was the Armstrong strain, which was grown in BHK cells (26). The WR strain of VV was grown in L-929 cells. The Indiana strain of vesicular stomatitis virus (VSV) was used in the IFN assays.
Infection of target cells. MEF were dispensed in 25 cm² plastic flasks (Falcon; Oxnard, CA), and were infected with MCMV at a multiplicity of infection (MOI) of 3-5 and used in cytotoxicity assays 3 days later. Greater than 99% of the cells were infected, as judged by cytopathic effect (CPE). With LCMV, the MOI was 0.005-.05 and the cells were used in cytotoxicity assays 3 days post-infection, when they were fully susceptible to LCMV-specific T cell-mediated lysis. Cells were infected with VV at an MOI of 5, and used as targets 24 hr later.

Interferon pretreatment of target cells. Twelve to twenty-four hr before use in cytotoxicity assays, when > 99% of MCMV- or VV-infected MEF had CPE, or when about 80% of LCMV-infected MEF were susceptible to LCMV-specific T cell-mediated lysis, beta interferon (Lee Biomolecular, San Diego, CA) was added to the culture fluid at 10,000 U/ml. These cells, along with untreated control cells, were then washed and labeled as described in the next section.

Cytotoxicity assay. Assay medium was RPMI-1640 medium supplemented with 0.1 M N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) (Sigma Chemical Co., St. Louis, MO), 10% FBS, glutamine, and antibiotics. The assay was performed as described (16). Briefly, target cells labeled with 100 uCi sodium chromate (New England Nuclear, Boston, MA) for 1 hr at 37°C were washed and mixed with various numbers of effector cells in round-bottomed microtiter wells at 5 X 10³ target cells/well. For spontaneous release determinations, medium was added to the wells, and 1 % Nonidet P-40 was added for maximum release determinations. Plates were incubated for 4 to 20 hr at 37°C in a humidified atmosphere
of 5% CO₂ and 95% air. At the end of the incubation, plates were centrifuged at 200 × G for 5 min, and 0.1 ml of the supernatant was collected and counted for radioactivity in a Beckman Gamma 5500 counter (Beckman Instruments, Palo Alto, CA). Data are expressed as percent specific release:

\[
100 \times \frac{\text{cpm experimental} - \text{cpm spontaneous}}{\text{cpm maximum} - \text{cpm spontaneous}}
\]

Spontaneous release was between 10 and 38%. Standard deviations of quadruplicate replica samples were less than 10% of the mean and were not listed in the tables.

**Activation of effector cells.** C57BL/6 mice were injected i.p. with 3 × 10⁵ PFU salivary gland MCMV, and spleen cells were isolated the next day (10). For LCMV, the dose was 8 × 10⁴ PFU, and the spleen cells were used 3 days later (16).

**Antisera.** Rabbit antiserum to asialo GM₁ was purchased from Wako Chemicals, USA, Inc., Dallas, TX. This antiserum has previously been shown to selectively deplete NK cell activity in vivo and in vitro, (27-30). To deplete NK cell activity in vivo, anti-asialo GM₁ was diluted 1:10 in RPMI medium and given i.v. in a volume of 0.2 ml, 4-6 hr before challenge with virus. Anti-asialo GM₁ was used in vitro at a final dilution of 1:20. Monoclonal anti-thy 1.2 antibody was provided by Dr. Edward Clark, Genetic Systems Corp., Seattle, WA and used at a final dilution of 1:150. Monoclonal anti-Ly 5.1 antibody, clone M1/89, a gift from Dr. Eric Martz, University of Massachusetts, Amherst, MA (31), was used at a final dilution of 1:10. For in vitro treatment of spleen cells, 2-2.5 × 10⁷ spleen leukocytes were suspended
in 100 μl antibody and incubated for 20-30 min at room temperature. Twenty-five μl guinea pig serum diluted 1:2, a source of C', was then added, and the mixture was further incubated at 37°C for 45 min. The cells were then washed twice and resuspended in assay medium for use as effectors in cytotoxicity assays. Rabbit anti-mouse beta interferon (Lee Biomolecular) was first absorbed with MEF, and then used at a final dilution of 1:20 in cytotoxicity assays.

**Interferon assay.** Samples of culture fluids were titrated by twofold serial dilutions in 96-well, flat-bottomed microtiter plates, to which L-929 cells were added at 3 X 10^4 cells/well. Eighteen to 24 hr later, the wells were expressed as the log_2 of the highest reciprocal dilution resulting in 50 % reduction in CPE.

**Virus titration.** Spleen virus titers were determined by using 10% homogenates of tissue taken from individual mice. LCMV was quantitated by plaque assay on Vero cells, which are nonpermissive for MCMV growth. Inclusion of MCMV in LCMV plaque assays had no effect on the number of LCMV plaques. MCMV was quantitated by plaque assay on MEF. With spleen homogenates containing both viruses, LCMV was neutralized with guinea pig anti-LCMV serum before quantitation of MCMV. This antiserum had no effect on MCMV titers. Results are expressed as the geometric mean titer, i.e., the arithmetic averages of the logs of four separate animals titrated for virus individually + standard error of the mean (SEM). The P values represent the significance of the differences of the means between the designated sample and the normal, non-antibody-treated control, and were calculated using Student's t-test.
Results

Lysis of MCMV-infected MEF by spleen cells from MCMV or LCMV-infected mice. Spleen cells from mice infected with either MCMV or LCMV were more capable of lysing MCMV-infected MEF, uninfected MEF, or YAC-1 cells than were control spleen cells (10) (Table 1). This lysis was not virus-specific, as LCMV-activated NK cells were quite capable of lysing MCMV-infected MEF, as well as uninfected MEF and YAC-1 cells (Table 1). Throughout this study, we consistently observed that LCMV-induced NK cells lysed target cells more efficiently than MCMV-induced effectors, regardless of the target cell type used in the assay. Another consistent finding was that MCMV-infected MEF were more resistant than uninfected MEF to lysis by activated NK cells (Table 1).

Characteristics of MCMV-activated killer cells. Though others (10,17,19,) had determined that MCMV-activated effector cells had some characteristics of NK cells, definitive characterization with currently available reagents was lacking. Whereas treatment of effectors with anti-thy 1.2 and C' had no effect on target lysis (10) (Table 2 A), addition of anti-asialo GM₁ or anti-Ly 5.1 in the presence of C' significantly reduced spleen cell-mediated lysis of all three targets (Table 2 B). Spleen cells from MCMV-infected anti-asialo-GM₁-treated mice also did not lyse MEF, MCMV-infected MEF, or YAC cells (Table 2 C). Taken together, these data characterize the MCMV-activated spleen effector cells as NK cells. Note again that MCMV-infected MEF tended to be more resistant than uninfected MEF to activated NK cells.
Effect of NK cell depletion on virus titers in mice simultaneously infected with MCMV and LCMV. Previous studies (12) showed that selective depletion of NK cell activity by injection of anti-asialo GM₁ antibody resulted in enhanced synthesis of MCMV, mouse hepatitis virus (MHV), and VV, but not LCMV in the spleen and liver. Whereas it is likely that the differences in the sensitivity of viruses to NK cells in vivo is at the target cell level, the possibility existed that MCMV, VV, and MHV induced an antiviral effector mechanism not induced by LCMV. In order to determine whether this antiviral selectivity remained when an "NK-sensitive" virus (MCMV) and an "NK-insensitive" virus (LCMV) are present in the same animal, we infected both control and NK cell-depleted mice with MCMV and LCMV simultaneously. The data in Table 3 show that NK cell depletion of these dually infected mice results in a 63-fold increase in spleen MCMV titers as compared to control dually infected mice, while having no effect on spleen LCMV titers. Thus, the results obtained in mice infected simultaneously with LCMV and MCMV (Table 3) were the same as those obtained in mice infected with either LCMV or MCMV (12) and argue against the hypothesis that an antiviral effector cell is induced by MCMV but not LCMV.

Lysis of MCMV-infected MEF by control spleen cells. Endogenous spleen cells were capable of lysing NK-sensitive YAC-1 cells in 4 hr cytotoxicity assays, but both uninfected and MCMV-infected MEF were lysed poorly by these effectors (Table 4). However, when the assay length was increased to 20 hr, the lysis of MCMV-infected MEF was substantially
higher than that observed after 4 hr, whereas the lysis of uninfected MEF remained unchanged (Table 4). This confirms previously published work (18). Table 4 shows that spleen cells from mice treated with anti-asialo GM₁ \textit{in vivo} were incapable of lysing YAC-1, MEF, or MCMV-infected MEF. In contrast to the results obtained with MCMV-infected MEF, LCMV-infected MEF were just as insensitive as uninfected MEF to lysis by endogenous spleen cells (Table 5).

Previous studies by Lee and Keller showed that enhanced lysis of MCMV-infected fibroblasts over uninfected cells is independent of IFN induction, as pretreatment of these effectors with actinomycin D, which inhibits IFN induction, had no effect on the enhanced lysis (18). In order to test this hypothesis more directly, we added antibody to mouse beta IFN to the culture fluid during cytotoxicity assays in order to examine its effect on lysis. Whereas untreated culture fluid from spleen cells and MCMV-infected MEF had 32 units/ml IFN after a 20 hr incubation, fluid containing anti-IFN antibody had less than 4 units/ml (the lower limit of detection in our assay). Table 6 shows that antibody treatment had no effect on the enhanced lysis of MCMV-infected MEF over uninfected MEF.

IFN protects LCMV-infected and uninfected but not MCMV- or VV-infected cells from NK cell-mediated lysis. Trinchieri and Santoli (21) showed that several types of human target cells could be protected from human NK cell-mediated lysis by IFN, but not if they were virus-infected. In order to examine whether or not this phenomenon is operative in the
murine CMV system, MCMV-infected or uninfected MEF were untreated or pretreated for 18-24 hr with IFN. Whereas IFN almost totally protected uninfected MEF from NK cell-mediated lysis, it had no protective effect on MCMV-infected MEF (Table 7). Thus, in contrast to the results obtained in the absence of IFN pretreatment of target cells, MCMV-infected MEF were significantly more sensitive to activated NK cell-mediated lysis than uninfected MEF when target cells were preconditioned with IFN (Table 7).

Results similar to those obtained with MCMV-infected cells were obtained using VV-infected cells. Table 8 shows that VV-infected cells are not more sensitive than uninfected cells to activated NK cells. However, IFN did not protect VV-infected MEF to the same extent as it protected uninfected cells from NK cell-mediated lysis. When cells were pretreated with IFN, VV-infected cells were lysed to a significantly greater extent than uninfected cells. Similar results were obtained using L-929 target cells (data not shown).

In contrast to the results obtained with MCMV-infected target cells, IFN pretreatment was just as effective at protecting LCMV-infected MEF as uninfected MEF, regardless of whether NK cells were activated by LCMV or MCMV infection (Table 9). Similar results were obtained using L-929 target cells (data not shown). This shows that IFN can protect uninfected and LCMV-infected MEF, and that the virus used to activate the NK cells does not affect this result. Despite the fact that LCMV-infected and uninfected MEF were almost totally
protected by IFN against NK cell-mediated lysis (Table 9), IFN treatment did not diminish their susceptibility to LCMV-specific cytotoxic T cells. Spleen cells taken 7 days postinfection and containing virus-specific T cells lysed 80% of infected and only 23% of uninfected MEF at an effector to target of 100:1 in a 16 hr assay.
Discussion

Several laboratories have reported NK-like effectors induced during acute MCMV infection (10,17,19), but depletion of activity by treatment with a reagent which selectively depletes NK cell activity (anti-asialo GM1 antibody, e.g.) has not been previously reported. Masuda and Bennett (17) reported that MCMV-induced spleen cells capable of killing YAC-1 cells were resistant to anti-NK antibody and C'. This is not surprising, since virus-induced NK cells are known to be more resistant to treatment with anti-NK antibody and C' (32,33). The data in Table 2 showing sensitivity to anti-asialo GM1 and anti-Ly 5.1 (27,34) and resistance to anti-thy 1.2 conclusively demonstrate that this effector is an NK cell.

The fact that NK cells play different roles in LCMV and MCMV infections could be due to the nature of the effector cells generated in each of these infections. For instance, it is possible that LCMV either suppresses or does not induce the generation of a particular type of NK cell needed to mediate antiviral effects, whereas MCMV does induce such antiviral NK cells. However, the results of our experiments using mice simultaneously infected with both viruses argues against these possibilities, as NK cells provided protection against MCMV, despite the presence of LCMV, against which NK cells provided no protection, despite the presence of MCMV. The results of these experiments reduce the likelihood that differences in NK cell generation or effector function account for differences seen during MCMV and LCMV.
infections. It is also unlikely that NK cells are mediating antiviral effects by secreting IFN. NK cell depletion results in a substantial increase in both IFN levels and virus titers 6-72 hr after challenge with MCMV, while having no effect on IFN levels and virus titers in mice challenged with LCMV (12,13), a virus whose synthesis is enhanced in mice depleted of IFN by treatment with antibody to IFN (35).

A mechanism whereby NK cells could eliminate virus while preserving normal tissue is by preferential lysis of virus-infected cells over uninfected cells. In support of this concept, the data presented here indicate that endogenous spleen cells are capable of lysing MCMV-infected MEF more efficiently than uninfected MEF, confirming the findings of others (18). Whereas Lee and Keller (18) had partially characterized the endogenous effector cell, they did not eliminate the cytotoxic activity by using a reagent which selectively depletes NK cell activity. This was a particularly important point, as we have characterized previously undescribed, non-NK effectors capable of preferentially lysing MHV-infected targets (36,37). Our present data clearly show that anti-asialo GM₁, a reagent which selectively depletes NK cell activity (27-30), eliminated the cytotoxicity. In contrast to the results with MCMV, LCMV-infected MEF were not lysed by endogenous NK cells. This could be one reason why NK cells do not play a role in limiting LCMV infection (12).

The fact that MCMV-infected MEF are lysed more efficiently than uninfected MEF by endogenous NK cells is possibly a result of NK cell activation during the course of the 20 hr incubation period (38).
However, this putative activation is probably not due to IFN, as increased lysis of MCMV-infected targets occurs even in the presence of actinomycin D, which inhibits IFN production (18). Our present data confirm this observation in a more direct manner, as specific elimination of IFN with an antibody had no effect on lysis. This putative activation could be mediated by MCMV-induced glycoproteins, as is the case with measles virus (39). It is unlikely that the reason for the sensitivity of MCMV-infected MEF to endogenous NK cells in long assays is enhanced binding or "recognition" of targets by NK cells, as MCMV-infected MEF were actually less sensitive than uninfected MEF to lysis in short (4 hr) assays by NK cells activated in vivo by MCMV or LCMV infection. We have observed this phenomenon in other virus systems (20), and there are a number of possible explanations for this observation. MCMV infection could inhibit the ability of target cells to bind to NK cells, as is the case with herpes simplex virus (HSV)-infected Vero cells (20). Another possibility is that binding ability remains intact, as for Sendai virus-infected L-929 cells (20), but that the lethal hit is not delivered effectively by the NK cell. This could be due to IFN protection of the virus-infected target, as IFN induced early in the infection may be capable of protecting some virus-infected target cells before the viral replicative cycle has progressed far enough to alter host protein and RNA synthesis, processes which are necessary for IFN protection (21). However, the fact that we did not detect IFN in our MCMV-infected MEF cultures makes this less likely.
When MEF were infected with MCMV for two days with a high MOI (3-5), they could not be protected from NK cell-mediated lysis by addition of $10^4$ U/ml of exogenous IFN, a dose which almost totally protected uninfected and LCMV-infected MEF (Tables 5 and 7). The reason for these differences may be that MCMV infection causes CPE and alters cellular metabolic processes (40,41), one of which may be necessary for IFN protection, while LCMV infection causes little CPE and does not affect the levels of cellular protein and RNA synthesis (23). VV-infected cells, whose synthesis of host cell proteins is shut down, are not protected by IFN (21) (Table 6). IFN treatment, however, did not render LCMV-infected MEF insensitive to all types of cell-mediated lysis, as we have shown that IFN pretreatment actually increased by 2-3 fold the sensitivity of these LCMV-infected MEF to lysis by LCMV-specific cytotoxic T cells.

The observation that target cells could be protected from NK cell-mediated lysis by IFN was shown to be operative in vivo by Welsh and coworkers (22,42). They found that thymocytes or tumor cells isolated from virus-infected or IFN-treated mice are protected against NK cell-mediated lysis in vitro (42). Also, tumor cells pretreated in vitro with IFN and then injected into mice were rejected more slowly than untreated cells (22).

From these data, one could put forth a theoretical model explaining how NK cells may exert antiviral effects in vivo while preserving normal tissue. Early in infection, endogenous NK cells may become locally activated in an area having many MCMV- or VV-infected cells.
This activation could be directly at the cell surface, perhaps via viral glycoproteins, or it could be caused by IFN produced locally. Though some uninfected cells might be killed by this indiscriminate process, NK cell-mediated lysis would be taking place in an area having a relatively high percentage of infected cells, and damage to normal tissue would be localized and minimal. If the infection becomes more systemic, high levels of IFN would be induced (8), and IFN-induced NK cell activation (8,10) and proliferation (43) would take place. Whereas normal cells would be protected by IFN against lysis by these activated NK cells (21), MCMV- or VV-infected cells would not receive protection, and would therefore be lysed. During an LCMV infection, endogenous NK cells would not preferentially lyse LCMV-infected cells by an IFN-independent activation mechanism (16), and later in the infection, when high levels of IFN are induced (1), both uninfected and LCMV-infected cells would be protected from activated NK cell-mediated lysis. If these mechanisms were operative, one would expect that NK cells would protect a host against MCMV or VV, but not LCMV. Direct evidence exists for a protective role of NK cells against MCMV and VV, but not LCMV, as selective depletion of NK cell activity in vivo enhances MCMV and VV infection, while having no effect on LCMV infection (12) (Table 3).
Summary

Spleen effector cells induced early during acute murine cytomegalovirus (MCMV) infection were characterized as natural killer (NK) cells by their sensitivity to treatments with anti-asialo GM₁ and anti-Ly 5 antisera in the presence of C' and their lack of sensitivity to anti-thy 1.2 and C' treatment. Selective depletion of NK cell activity by injection with anti-asialo GM₁ antibody rendered mice more susceptible to infection with MCMV, but not lymphocytic choriomeningitis virus (LCMV) when mice were simultaneously infected with the two viruses, suggesting that NK cell-mediated antiviral effects may depend on target cell susceptibility to NK cell-mediated lysis rather than the ability of a virus to induce a specialized antiviral NK cell. In support of this concept, in long (20 hr) cytotoxicity assays, endogenous NK cells lysed mouse embryonic fibroblasts (MEF) infected with MCMV much more efficiently than LCMV-infected or uninfected MEF. The duration of the assay suggests an effector activation, but addition of antibody which neutralized the interferon (IFN) induced during cytotoxicity assays had no effect on lysis. This difference in lysis was probably not a result of greater sensitivity of these MCMV-infected MEF to lysis, as these targets were less sensitive than uninfected MEF to lysis by MCMV- or LCMV-activated NK cells in short-term 4 hr assays. When MEF and MCMV-infected MEF were pretreated with IFN, activated NK cell-mediated lysis of MCMV-infected MEF was undiminished and was much higher (up to 4-fold higher) than that of uninfected MEF, whose sensitivity to NK cell-mediated lysis was almost totally abolished by IFN pretreatment. IFN also failed to fully protect cells infected with
vaccinia virus (VV) against NK cell-mediated lysis. In contrast to the results obtained with MCMV or VV, LCMV-infected MEF were almost totally protected by IFN against LCMV or MCMV-activated NK cell-mediated lysis. These data could explain how NK cells might selectively eliminate MCMV- or VV-infected but not LCMV-infected or uninfected cells during acute infection in vivo, and why NK cells may play a role in resistance to MCMV and VV, but not LCMV.
References


Table 1

Lysis of MCMV-Infected and Uninfected MEF and YAC-1 Cells by Spleen Cells From MCMV- or LCMV-Infected Mice

<table>
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<tr>
<th>Effector Cells</th>
<th>E:T</th>
<th>(MCMV)</th>
<th>MEF</th>
<th>YAC-1</th>
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<td>MCMV-activated(^a)</td>
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<td>26.</td>
<td>56.</td>
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<td></td>
<td>33</td>
<td>11.</td>
<td>15.</td>
<td>36.</td>
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<tr>
<td></td>
<td>11</td>
<td>5.6</td>
<td>7.3</td>
<td>20.</td>
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<tr>
<td>LCMV-activated</td>
<td>100</td>
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<td>38.</td>
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<tr>
<td></td>
<td>11</td>
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<td>11.</td>
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</table>

\(^a\)Spleen cells from MCMV or LCMV-activated C57BL/6 mice 3 days postinfection were prepared as described in Materials and Methods and used as effectors in 4 hr cytotoxicity assays.
### Table 2
Characteristics of MCMV-Activated Killer Cells

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<th>Exp.</th>
<th>Treatment</th>
<th>percent specific $^{51}$Cr release</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(MCMV)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>none</td>
<td>23.</td>
</tr>
<tr>
<td></td>
<td>C'</td>
<td>19.</td>
</tr>
<tr>
<td></td>
<td>anti-thy 1.2 + C'</td>
<td>16.</td>
</tr>
<tr>
<td>B</td>
<td>none</td>
<td>12.</td>
</tr>
<tr>
<td></td>
<td>C'</td>
<td>11.</td>
</tr>
<tr>
<td></td>
<td>anti-Ly 5 + C'</td>
<td>5.7</td>
</tr>
<tr>
<td></td>
<td>anti-AGM$_1$ + C'</td>
<td>-0.4</td>
</tr>
<tr>
<td>C$^2$</td>
<td>none</td>
<td>8.9</td>
</tr>
<tr>
<td></td>
<td>anti-AGM$_1$</td>
<td>-1.5</td>
</tr>
</tbody>
</table>

$^1$Spleen cells from MCMV-infected C57BL/6 mice were treated with various antisera and C', as described in Materials and Methods, and used in 4 hr cytotoxicity assays. Effector to target ratios were 100:1 in exp. A, B, and 50:1 in exp. C.

$^2$Anti-asialo GM$_1$ (anti-AGM$_1$) was given 4-6 hr before virus challenge.
Table 3
Effect of NK Cell Depletion on Spleen Virus Titers in Mice Infected Simultaneously with LCMV and MCMV

<table>
<thead>
<tr>
<th>anti-asialo GM$_1$</th>
<th>percent NK lysis</th>
<th>log$_{10}$ pfu/spleen</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>62.0</td>
<td>3.0 ± 0.2</td>
</tr>
<tr>
<td>+</td>
<td>0.1</td>
<td>4.8 ± 0.1$^b$</td>
</tr>
</tbody>
</table>

$^a$Groups of 4 C57BL/6 mice were either left untreated or treated with anti-asialo GM$_1$, as described in Materials and Methods. Four to 6 hr later, each mouse was infected simultaneously by i.p. injection of $8 \times 10^4$ PFU of LCMV and $7 \times 10^4$ PFU of MCMV. Three days later, the mice were sacrificed, and pooled spleen cells were assayed for NK cell activity against YAC-1 cells at 100:1 E:T; assay length was 6 hr. Spleen virus titers were determined by plaque assay, as described in Materials and Methods.

$^b$P < .001, as compared to non-antibody-treated controls.

$^c$Not significantly different from non-antibody-treated controls.
Table 4

Lysis of MCMV-Infected and Uninfected MEF and YAC-1 Cells by Spleen Cells From Control or Anti-Asialo GM₁-Treated Mice

<table>
<thead>
<tr>
<th>Assay length</th>
<th>Treatment</th>
<th>E:T</th>
<th>MEF (MCMV)</th>
<th>MEF</th>
<th>YAC-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 hr&lt;sup&gt;a&lt;/sup&gt;</td>
<td>none</td>
<td>100</td>
<td>9.3</td>
<td>2.8</td>
<td>18.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>33</td>
<td>4.2</td>
<td>-0.1</td>
<td>9.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>11</td>
<td>0.9</td>
<td>-0.3</td>
<td>5.0</td>
</tr>
<tr>
<td></td>
<td>anti-AGM₁</td>
<td>100</td>
<td>0.8</td>
<td>-1.7</td>
<td>2.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>33</td>
<td>1.6</td>
<td>-0.8</td>
<td>-0.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>11</td>
<td>0.9</td>
<td>-1.5</td>
<td>0.5</td>
</tr>
<tr>
<td>20 hr</td>
<td>none</td>
<td>100</td>
<td>28.</td>
<td>1.2</td>
<td>38.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>33</td>
<td>11.</td>
<td>0.5</td>
<td>18.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>11</td>
<td>4.4</td>
<td>1.2</td>
<td>-4.3</td>
</tr>
<tr>
<td></td>
<td>anti-AGM₁</td>
<td>100</td>
<td>2.3</td>
<td>-1.2</td>
<td>-2.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>33</td>
<td>1.0</td>
<td>-4.6</td>
<td>-0.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>11</td>
<td>-4.5</td>
<td>-3.7</td>
<td>-1.5</td>
</tr>
</tbody>
</table>

<sup>a</sup>Spleen cells from control or anti-asialo GM₁ (anti-AGM₁)-treated C57BL/6 mice were used as effectors in cytotoxicity assays performed as described in Materials and Methods.
Table 5

Lysis of LCMV-Infected and Uninfected MEF and YAC-1 Cells by Endogenous Spleen Cells

<table>
<thead>
<tr>
<th>Assay length</th>
<th>E:T</th>
<th>percent specific $^{51}$Cr release</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MEF (LCMV)</td>
<td>MEF</td>
</tr>
<tr>
<td>4 hr&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>0.1</td>
<td>-2.9</td>
</tr>
<tr>
<td>50</td>
<td>-2.9</td>
<td>-0.6</td>
</tr>
<tr>
<td>25</td>
<td>0.9</td>
<td>2.5</td>
</tr>
<tr>
<td>20 hr</td>
<td></td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>2.7</td>
<td>1.9</td>
</tr>
<tr>
<td>50</td>
<td>2.7</td>
<td>2.4</td>
</tr>
<tr>
<td>25</td>
<td>0.1</td>
<td>1.0</td>
</tr>
</tbody>
</table>

<sup>a</sup>Spleen leukocytes from control C57BL/6 mice were used as effectors in cytotoxicity assays performed as described in Materials and Methods.
Table 6
Anti-IFN Antibody Does Not Affect Lysis of MCMV-Infected or Uninfected MEF by Endogenous NK Cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>E:T</th>
<th>MEF (MCMV)</th>
<th>MEF</th>
<th>YAC-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>none(^a)</td>
<td>100</td>
<td>19.</td>
<td>10.</td>
<td>35.</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>11.</td>
<td>4.3</td>
<td>23.</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>7.2</td>
<td>3.3</td>
<td>16.</td>
</tr>
<tr>
<td>Anti-IFN(^b)</td>
<td>100</td>
<td>17.</td>
<td>9.4</td>
<td>34.</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>10.</td>
<td>4.5</td>
<td>22.</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>4.7</td>
<td>3.7</td>
<td>12.</td>
</tr>
</tbody>
</table>

\(^a\)Cytotoxicity assays were performed as described in Materials and Methods; assay length was 18 hr.

\(^b\)Anti-IFN antibody (Anti-IFN) was included in the assay at a final dilution of 1:20.
Table 7

Interferon Protects Uninfected But Not MCMV-Infected MEF From Activated NK Cell-Mediated Lysis

<table>
<thead>
<tr>
<th>Assay Length</th>
<th>E:T</th>
<th>percent specific (^{51})Cr release</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>MEF (MCMV)</td>
</tr>
<tr>
<td>4 hr(^a)</td>
<td>200</td>
<td>12.</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>8.3</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>5.3</td>
</tr>
<tr>
<td>8 hr</td>
<td>200</td>
<td>19.</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>13.</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>7.4</td>
</tr>
</tbody>
</table>

\(^a\)Spleen effector cells were from MCMV-infected C57BL/6 mice. Target cells were left untreated or pretreated with IFN as described in Materials and Methods, then used in cytotoxicity assays.
Table 8
Reduced Ability of Interferon to Protect Vaccinia-Infected Cells From LCMV-Activated NK Cell-Mediated Lysis\(^a\)

<table>
<thead>
<tr>
<th>Target Cell</th>
<th>Effector:Target</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>100</td>
</tr>
<tr>
<td>MEF (VV)</td>
<td>31.</td>
</tr>
<tr>
<td>MEF (VV) IFN</td>
<td>20.</td>
</tr>
<tr>
<td>MEF</td>
<td>32.</td>
</tr>
<tr>
<td>MEF IFN</td>
<td>11.</td>
</tr>
</tbody>
</table>

\(^a\)Spleen cells from LCMV-infected C3H/St mice were used as a source of NK cell effectors. Assay length was 6 hr. Results are expressed as percent specific \(^{51}\)Cr release.
<table>
<thead>
<tr>
<th>Effector cells</th>
<th>E:T</th>
<th>MEF (LCMV)</th>
<th>IFN (MEF)</th>
<th>MEF (LCMV)</th>
<th>IFN (MEF)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>100</td>
<td>42.</td>
<td>9.1</td>
<td>45.</td>
<td>10.</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>28.</td>
<td>6.7</td>
<td>31.</td>
<td>6.3</td>
</tr>
<tr>
<td>MCMV-activated</td>
<td>200</td>
<td>29.</td>
<td>12.</td>
<td>29.</td>
<td>11.</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>21.</td>
<td>8.4</td>
<td>21.</td>
<td>7.3</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>17.</td>
<td>5.5</td>
<td>16.</td>
<td>6.0</td>
</tr>
</tbody>
</table>

^aLCMV or MCMV-activated spleen cells from C57BL/6 mice were used as effectors. Target cells were left untreated, or pretreated with IFN, as described in Materials and Methods, then used in cytotoxicity assays.
CHAPTER 3

Discussion

The Antiviral Role of NK Cells in Vivo

The fact that NK cells are capable of mediating tumor surveillance has been well established (reviewed in ref. 77 and in introduction). It is not yet known what effects, if any, NK cells have against bacteria, fungi, protozoa, or yeast, but much circumstantial evidence (see introduction) favors a role for NK cells against certain virus infections, although this evidence has been far from conclusive.

Direct evidence for an antiviral role of NK cells had been lacking, due to the absence of methods for specifically depleting NK cell activity in vivo. Antibody to asialo GM1, a neutral glycosphingolipid present at high quantities on NK cells, selectively depletes NK cell activity in vivo, while having no detectable effect on other immune functions (2,14,20,78). Using this reagent, we have shown that NK cell depletion results in up to 1000-fold increases in MCMV, MHV, and VV titers (II,III) in the spleens and livers of acutely infected mice 3 days postinfection, before specific immune responses such as CTL are detectable (48). Correlating with increased virus synthesis, these NK cell-depleted, virus-infected mice also had more extensive hepatitis (II,III-Fig.1) and spleen necrosis (III-Fig.1) than virus-infected, control mice.
In agreement with expectations based on previous data (50, 52), NK cell depletion had no effect on the course of acute (II-Table 2) or persistent (II-Tables 3 and 4) LCMV infection. These data, although not totally conclusive, provided the most convincing evidence to date that NK cells were capable of mediating antiviral effects in vivo, and that their importance varied depending on the virus. Anti-asialo GM1 does not affect any known immune functions other than NK cell function, but it does react with some monocytes and thymocytes (20), and could have unknown effects on some yet undefined resistance mechanisms.

Because of these uncertainties, we performed adoptive transfer studies using MCMV as a model system. Using a variety of physical and immunochemical techniques to enrich and deplete NK cell activity we have, for the first time, obtained definitive evidence that NK cells are capable of mediating antiviral effects in vivo (V). The results showed that the cell population mediating the resistance had the phenotype of an NK cell: a nylon wool nonadherent, asialo GM1+, NK 1.2+, Ly 5+, Thy-1-, Ia-, low-density lymphocyte (V-Table 3). Further, IL-2-dependent cloned NK cells (but not T cells) were able to provide resistance, showing that no other adoptively transferred cell population was necessary to obtain resistance (V-Table 1 and Figs. 4 and 5).

Selgrade and Osborn (68), using the same adoptive transfer system as we used, showed that either thioglycollate-stimulated peritoneal macrophages or endogenous, macrophage-depleted spleen leukocytes could enhance survival in MCMV-infected suckling mice.
However, the nature of the spleen leukocytes responsible for the resistance was unknown. Johnson (69) suggested that adult but not suckling macrophages might provide resistance to herpes simplex type 1 (HSV-1), and Hirsch et al. (70) showed that adoptive transfer of adult macrophages enhanced survival of HSV-1-infected suckling mice, but characterization of the cells responsible for protection was not carried out with currently available reagents. Tardieu et al. (40) used a similar adoptive transfer system to demonstrate that transfers of adult unstimulated T cells, macrophages, and bone marrow cells could enhance survival of MHV-infected suckling mice. Again, the nature of the bone marrow cells providing protection was unknown, but bone marrow cells from mice less than 3 weeks of age did not protect, and this corresponds to the age at which NK cell activity begins to develop. Our current data regarding MCMV and MHV provide strong evidence that these investigators were observing the antiviral effects of NK cells in their studies involving adoptive transfers of spleen leukocytes and bone marrow cells. Our current studies with MCMV definitively show that adult macrophages are not required for resistance of suckling mice to MCMV. Naive adult spleen cells depleted of macrophages by nylon wool passage (V-Table 1) or by size separation (V-Fig.2) still protected against MCMV. Treatments which depleted NK cell activity but left macrophages intact, such as anti-asialo GM1 (V-Table 1 and Fig.2), anti-NK 1.2 (V-Table 1), and anti-Ly 5 (V-Fig.2) and complement destroyed the protective capacity of adult spleen cells. As stated previously, adult mice treated with anti-asialo GM1, a treatment which leaves
macrophage function intact (20, 78), were much more susceptible to MCMV and MHV infection, again showing that NK cells were mediating resistance. Habu and Okumura (79) have also shown that use of this antibody lowers resistance to HSV-1. To summarize, our data do not disprove that macrophages could be providing some protection, but for the first time, we show that NK cells depleted of macrophages can provide resistance against a virus infection (V).

It has been known for over 10 years that T cells have antiviral effects in vivo (73). It is recognized that T cells are responsible for clearing virus (48, 73, 80), and recovery from illness caused by virus infections is attributed to T cells (81). However, virus-specific T cells are not detected until 4-10 days postinfection (32, 48), and are thus unlikely to contribute to host resistance during the first few days of acute virus infection of a nonimmune host. Our evidence indicates that NK cell-mediated protection against morbidity and mortality early during acute infection (3-5 days postinfection) takes place without the participation of T cells (III, V-Figs. 2 and 3). Athymic nude mice depleted of NK cells by injection with anti-asialo GM1 synthesized up to 6 times more MCMV in their organs as compared to nude mice not receiving antibody (V-Fig. 3). Adult spleen cells depleted of T cells by treatment with monoclonal anti-thy 1.2 and complement still protected suckling mice against lethal MCMV infection (V-Fig. 2), so transfer of T cells is not essential to transfer of resistance.

There has been little evidence to implicate neutrophils in resistance to virus infections. Our adoptive transfer experiments
show that populations of lymphocytes with greater than 98 percent purity are quite capable of mediating resistance (V-Fig.2).

Although virus-specific antibody can be detected as early as 3 days postinfection (49), the role of antibody in virus infections is thought to be one of protection against reinfection by the same virus subsequent to recovery from that same virus (81), as peak antibody titers are most often seen after the recovery phase (82,83). Our experiments show that depletion of B cells by treatment with anti-Ia and complement (V-Fig.2), or by nylon wool passage (V-Table 1) did not deplete the protective capacity of adoptively transferred spleen cells.

Our survival curves indicated that $5 \times 10^7$ adult spleen cells could prolong survival of MCMV-infected mice, but that 3-fold fewer cells could not (V-Fig.2). If the protection were mediated by NK cells, then 3-fold fewer spleen cells with 3 times as much NK cell activity should protect. This prediction was supported by our data, as $1.7 \times 10^7$ unfractionated spleen cells did not fully protect, while $1.7 \times 10^7$ spleen cells enriched 3-fold for NK cell activity provided as much protection as $5 \times 10^7$ unfractionated spleen cells (V-Table 1).

Previous work using an IL-2-dependent cloned NK cell line derived from C57BL/6 mice (84) showed that these cells were capable of mediating resistance to tumor implants and bone marrow transplants (85). These cells are large granular lymphocytes with the phenotype asialo GM1$^+$, NK 1.2$^+$, Ly 1$^-$, Ly 2$^-$, thy-1$^+$ and are capable of mediating in vitro lysis of NK-sensitive YAC-1
tumor cells (84). Our results clearly show that these cells are capable of providing protection against MCMV (V-Table 1 and Fig.1), but not LCMV (V-Table 2). In contrast, a cloned T cell line also derived from C57BL/6 mice provided no protection, indicating that cloned lymphoid cells in general do not necessarily protect.

Because data for survival curves must be accumulated over a period of 2-3 weeks, it is possible that over that period of time subpopulations of adoptively transferred spleen cells could differentiate and provide resistance, making it difficult to tell which type of cell is providing the resistance. Previous work by Shellam et al. (46) showing that radiation bone marrow chimeras created by transferring donor bone marrow cells into irradiated recipients reflected the MCMV resistance patterns and NK cell activities of the donors was subject to this criticism, as recipient mice were not challenged with virus until 8 weeks after adoptive transfer. Our present studies showing that adoptively transferred spleen cells containing NK cell activity result in reduced virus titers 3 days postinfection (V-Table 1) lowers the possibility that a subpopulation of these cells differentiated inside the recipient and subsequently provided protection. This is especially true when referring to the specific immune response (CTL and antibody) which takes 6-15 days to fully develop. Even more definitive in this regard are the data obtained with adoptive transfer of cloned NK cells, which reduced day 3 virus titers up to 500-fold (V-Table 1).

We do not know if adoptively transferred NK cells (given i.p.) are capable of populating recipient spleens. Our unpublished data
indicate that recipient spleen NK cell activity is not changed by adoptive transfer of NK cells whether the recipient is left uninfected or challenged with LCMV. Peritoneal NK cell activity of recipient mice was not examined. However, NK cell-deficient suckling mice receiving NK cells followed the next day by MCMV challenge had more spleen leukocytes and greater NK cell activity than MCMV-challenged mice receiving no NK cells. This result could be explained by a repopulation of the recipient spleen by donor cells (which did not occur in uninfected or LCMV-infected recipients), but is more likely a result of NK cell-mediated antiviral effects, possibly occurring in the peritoneum, which is the site of both virus inoculation and NK cell transfer. Reduced MCMV titers in the peritoneum could lead to reduced spread of virus to the spleen, reducing the spleen leukopenia and inhibition of NK cell activity associated with high spleen MCMV titers (46). A third alternative is that donor cells could be triggering recipient antiviral mechanisms in the peritoneum, which act at a distant site, e.g. inside the spleen. Finally, donor NK cells may be populating the recipient spleen, but their activity may be undetectable using in vitro cytotoxicity assays.

The Role of NK Cells During Persistent Virus Infection

The persistent phase of a virus infection is generally defined as a period of time after the acute phase during which infectious virus can be isolated from the host. A balance between the virus
and the host's defense mechanisms has been struck: the host does not eliminate the virus, yet the ability of the virus to act in an immediately harmful way is held in check by host defense mechanisms, many of which remain undefined. I will limit the discussion to persistent infections in the mouse involving LCMV and MCMV.

Persistent LCMV infection has been studied extensively, yet the mechanism of persistence still remains unknown. Establishment of persistent LCMV infection can be accomplished by inoculating newborn mice (86) or immunosuppressed adult mice (73) with LCMV. Mice infected in utero or at birth carry LCMV for life, and the virus is present in virtually every organ, though mice often do not show overt disease symptoms (86). Early studies (87) concluded that no specific immune response (T or B cell) was generated to LCMV, but the classic studies of Oldstone and Dixon (88) demonstrated that virus-specific antibody exists in immune complexes composed of antibody-virion aggregates, which probably lead to chronic immune complex disease (90). A T cell response has never been demonstrated during persistent infection (76), but adoptive transfer of virus-specific CTL causes a marked reduction in virus titers, but not total elimination of the virus (91).

Extensive studies on our part (I-Table 1) showed that these persistently infected mice have abnormally high levels of NK cell activity as compared to uninfected controls. This NK cell activity was elevated for at least 6 months (I-Table 1), well after the time period during which NK cell activity in control mice becomes extremely low (1,5). The most likely explanation for this high NK
cell activity is the fact that these mice have low levels (32-64 U/ml) of IFN type I in their plasma, which is known to activate NK cells. Our report (I-Table 4) and that of Saron et al. (92) were the first to show the presence of IFN in mice persistently infected with LCMV. The NK cells in these mice were not undergoing blastogenesis, but were capable of doing so when the mice were given poly I:C, a potent IFN inducer (I-Figs.1 and 2). It is still not known whether this elevated NK cell activity is a result of increased numbers of NK cells or increased lytic activity on a per cell basis.

Because persistently infected mice had elevated NK cell activity, we hypothesized that this activity may be keeping LCMV titers under control. Previous evidence (52) using different LCMV and mouse strains argued against a role for NK cells in controlling this infection, as cyclophosphamide treatments, which reduce NK cell activity, did not alter LCMV titers in these persistently infected mice. However, it was not known whether or not the mice used in that previous study had elevated NK cell activity. Our results (II-Tables 3 and 4) showed that specific depletion of NK cells using antibody to asialo GMI had no effect on LCMV titers in the blood or the spleen. The reason for this lack of effect could be that LCMV-infected target cells are, under conditions prevailing in vivo, insensitive to NK cell-mediated lysis. This will be discussed in a subsequent section.

In contrast to persistent LCMV infection, the persistent phase of MCMV infection is transient and occurs after inoculation of
either newborn or immunocompetent adult mice (93,96). After resolution of acute infection, the virus persists in the spleen but more frequently in the salivary gland for several months, after which time it becomes undetectable, and has presumably become latent. During persistent or latent infection, CMV-specific CTL activity is undetectable, but antibody titers are at high levels. Treatment of these mice with cyclophosphamide or cortisone causes reactivation and dissemination of latent virus, without affecting antibody titers (95). Treatment of mice with cortisone during the persistent phase of the infection increases salivary gland MCMV titers while inhibiting the infiltration of lymphocytes into the salivary gland (82). Because drug-induced reactivation of latent virus and drug-induced increases in salivary gland virus titers during persistent MCMV infection take place in the face of high antibody titers and at a time when CTL activity is undetectable, we tested the hypothesis that NK cells may be limiting virus replication during the persistent phase of MCMV infection. Our results show that specific depletion of NK cells using antibody to asialo GM1 resulted in a 6-8-fold increase in salivary gland MCMV titers (III-Table 8). However, no dissemination of the virus to other organs was noted, and attempts to cause reactivation of latent MCMV infection using anti-asialo GM1 have been so far unsuccessful. Nonetheless, these experiments provide the first solid piece of evidence that NK cells may play a role in controlling persistent infection. It is noteworthy that NK cell depletion has no effect on salivary gland virus titers during acute MCMV infection, despite
causing major increases in spleen and liver virus titers during the acute phase (III-Table 1), and causing increases in salivary gland titers during the persistent phase (III-Table 8). A possible mechanism to explain this difference could be NK cell-mediated ADCC against MCMV-infected targets. This has been shown by Manischewitz et al. (98) to occur in vitro using serum from MCMV-infected mice 15-30 days postinfection, that is, after the acute phase but during the persistent phase of the infection. Perhaps NK cells can control salivary gland virus titers only by ADCC, and acute salivary gland infections are not inhibited by NK cells due to lack of MCMV-specific antibody, which reaches optimal titers at about 15 days postinfection (82,83). Thus, NK cells could be required for control of persistent MCMV infection, despite the existence of high antibody titers.

The Effect of NK Cell Depletion on Viral Pathogenesis

Susceptibility to virus infections varies among individuals and also changes within the same individual dependent upon such factors as genotype, age, nutritional status, and stress levels (77). These factors, some of which can change quickly, also affect NK cell activity. Our studies (III) describe the events whereby a lowered defense mechanism (i.e. NK cells) can lead to: 1) contraction of a viral disease which otherwise would be totally repelled or subclinical; 2) increased severity (possibly leading to death) of a viral disease which would otherwise be mild; 3) increased duration
of viral disease with delayed recovery.

We found that in some instances, control mice challenged with low doses of MCMV or VV were not clinically ill, had no detectable virus in their spleen and livers, and had no liver pathology at 3 days postinfection. In contrast, mice whose NK cells were depleted with anti-asialo GM1 were clinically ill (i.e. ruffled fur and hunched posture), had moderate amounts of virus in their spleens and livers, and exhibited pathological liver lesions (III). With higher doses of virus, NK cell-depleted mice at 3 days postinfection had increased mortality and morbidity, associated with massive spleen and liver necrosis (III-Fig.1), severe leukopenia (III-Table 3), and marked suppression of the T cell response (III-Table 2), as compared to control virus-infected mice.

NK cell-depleted mice also recovered more slowly from infection, as control mice had no detectable virus in their spleens, lungs, and livers at day 9 (III-Table 1), whereas NK cell-depleted mice did not clear the virus from these organs until 12-15 days postinfection. NK cell depletion at 0-2 days postinfection had a marked effect on viral disease, but NK cell depletion late in the infection (6-8 days) had no effect on MCMV synthesis or clinical illness (III-Table 4). Thus, the lack of NK cells at this time did not affect viral clearance, presumed to be a T cell-dependent function (48).

Thus, the lack of NK cells early in the infection can cause severe pathological consequences at day 3. Further, the resulting increased virus titers are likely the cause of the diminished T cell
function seen in these NK cell-depleted, virus-infected mice, as MCMV is known to suppress T cell function (99,100). This diminished T cell function (assayed by Con A response) probably results in delayed clearance of virus and thus a prolongation of disease.

Whereas NK cell depletion had a profound effect on the pathogenesis of MCMV infection after i.p or i.v. inoculation, the course of disease after intranasal inoculation was not affected by NK cell depletion (III-Table 7). Quinnan et al. (101) have shown that cortisone treatment of mice during intranasal MCMV infection results in higher spleen and lung virus titers, concurrent with lower NK cell activity. They concluded that NK cells in the lung were mediating antiviral resistance, but our results using a more specific reagent (anti-asialo GM1) for elimination of NK cell activity show that NK cell depletion is without effect (III-Table 7). NK cell-deficient beige mice did not undergo more severe MCMV infection after intranasal inoculation (III-Table 7). The reason why NK cells limit synthesis after i.p. and i.v. but not intranasal inoculation is not known, but the lung may have an antiviral mechanism distinct from that of NK cells. In support of this concept, Biron et al. (102) have demonstrated that a cortisone-sensitive non-NK, non-T cell is responsible for preferential rejection of virus-infected cells over uninfected cells from the lungs in 4 hour in vivo cytotoxicity assays.

Mechanism of NK Cell-mediated Antiviral Effects
There are several mechanisms whereby NK cells could mediate antiviral effects in vivo. NK cells are known to secrete IFN when stimulated with tumor cells, viruses, or plant lectins (103). The secreted IFN could then mediate antiviral effects by directly protecting cells against virus replication, or by modulation of the host response (e.g. activation of NK cells or macrophages. The mechanism of IFN-mediated antiviral action in vivo is currently the subject of much study, and will be discussed in detail later. However, evidence from our laboratory argues against an NK cell-derived IFN-mediated mechanism of antiviral action. We have shown that depletion of NK cells during acute infection with MHV, MCMV, and VV actually results in higher titers of IFN in the plasma and peritoneal wash, correlating with higher spleen and liver virus titers (II,III). These data indicate that NK cells are not major producers of IFN during virus infection.

Throughout our studies we have documented that NK cells do have antiviral effects against MCMV, VV, and MHV, but not LCMV (II,III,V). A possible reason for these differences could be that MCMV, MHV, or VV are causing induction of a certain type of NK cell capable of mediating antiviral effects whereas LCMV, which is quite capable of inducing activation and proliferation of NK cells (32), is not inducing antiviral NK cells, or is causing suppression of antiviral NK cell induction or effector function. Our results showing that NK cell depletion of mice infected simultaneously with MCMV and LCMV argues against this hypothesis. NK cell depletion resulted in a 63-fold increase in MCMV titers in the spleens of
these dually-infected mice, despite the presence of LCMV, as compared to control dually-infected mice, while having no effect on LCMV titers in these mice (VI-Table 3).

The results we have obtained with MCMV, VV, and LCMV suggest that the susceptibility of virus-infected targets to NK cell-mediated lysis in vitro correlates with NK cell-mediated antiviral effects in vivo. Whereas LCMV-infected or uninfected targets are not lysed by endogenous NK cells (32, VI-Table 5), MCMV-infected targets are lysed by these effectors (VI-Table 4). When target cells are pretreated with IFN, activated NK cells induced during acute virus infections do not lyse normal or LCMV-infected targets (VI-Table 9), but they do lyse VV- (VI-Table 8) or MCMV- (VI-Table 7) infected targets. These results support the hypothesis that NK cells mediate antiviral effects in vivo by lysing virus-infected cells, and that the failure of NK cells to lyse virus-infected cells results in their failure to mediate antiviral effects.

We do not know why endogenous NK cells are capable of lysing MCMV- but not LCMV-infected targets. The lysis is detectable during in vitro cytotoxicity assays lasting 18-20 hours, but not in assays of a 4 hour duration (VI-Table 4), indicating that the lytic process may require activation of the endogenous NK-like cells by the virus-infected targets. Lee and Keller first showed that NK cells lysed MCMV-infected cells during cytotoxicity assays and that this happened in the absence of IFN (41). Casali et al. (58) showed that measles virus glycoproteins were capable of activating NK cells
independently of IFN, so it is possible that MCMV glycoproteins on the infected target cell surface are responsible for activating NK cells. Kirchner et al. (104) have shown that i.p. injection of HSV-1 inactivated by ultraviolet light can activate mouse peritoneal NK cells within hours, while no IFN is detectable in the peritoneal fluid, suggesting that viral components themselves may be capable of activating NK cells in vivo, independent of IFN. In contrast, LCMV-infected target cells may not be capable of activating NK cells (32), resulting in the failure of these targets to be lysed.

MCMV-infected cells are not simply more sensitive than uninfected or LCMV-infected cells to lysis by NK cells, as NK cells already activated during acute LCMV or MCMV infection actually lyse MCMV-infected cells less efficiently than LCMV-infected or uninfected cells (36, VI-Table 1). This has been seen with several virus-cell systems (56), and the reason for it is unknown, but could be due to reduced binding of NK cells to MCMV-infected targets, or MCMV-induced alterations in the target cell membrane resulting in a reduced ability of the target cell to trigger the NK cell lytic mechanism. IFN is probably not protecting MCMV-infected target cells in our system (fibroblast targets), because IFN is not detectable in our MCMV-infected fibroblast cultures.

Trinchieri et al. first showed that exogenously added IFN can protect normal cells but cannot protect VV- or influenza virus-infected cells against NK cell-mediated lysis, nor can it protect cells whose protein and RNA synthesis has been inhibited by cyclohexamide and actinomycin D, respectively (61). Cytopathic
viruses such as VV, MCMV, and influenza alter host protein and RNA synthesis (105), processes required for IFN-mediated protection, and therefore, cells infected with these viruses are left susceptible to NK cell-mediated lysis. In contrast, LCMV is a relatively non-cytopathic virus (106), and most host cell functions are left intact. As a result, IFN protects these LCMV-infected cells, and these cells escape NK cell-mediated lysis.

From these data, one could put forth a theoretical model explaining why NK cells play a role against MCMV and VV, but not LCMV. During the initial phase of a VV or MCMV infection, virus-infected cells may activate NK cells via their viral glycoproteins, or by locally inducing IFN. NK cell-mediated lysis would therefore only occur in areas where virus-infected cells were found. As the infection progresses, high levels of IFN are induced, activating NK cells and protecting normal tissue against NK cell-mediated destruction, but leaving MCMV- or VV-infected cells susceptible to lysis. In contrast, during an LCMV infection, endogenous NK cells would not be activated by LCMV glycoproteins, leaving LCMV-infected cells intact. As the infection progressed high levels of IFN would activate NK cells and protect normal cells as before, but LCMV-infected cells would be protected as well. These data could thus explain why NK cells play a role against MCMV and VV infections, but not LCMV infection.
It is well established that endogenous IFN is very important in providing resistance to virus infections. The most extensive studies in this area have been done by Gresser and his colleagues (64,65), using antibody to IFN. Injection of mice with this antibody before infection with HSV-1, VSV, Newcastle disease, Moloney sarcoma, and encephalomyocarditis viruses greatly increases the severity of these infections. However, the issue of whether IFN is mediating direct antiviral effects or acting through early nonspecific defense mechanisms such as NK cells or macrophages is still not resolved. I will restrict the discussion to interactions between IFN and NK cells, and later between IFN and T cells. Because IFN causes activation and blastogenesis of NK cells (32,34), it is likely that IFN could mediate antiviral effects by its action on NK cells.

The most clearcut evidence for an antiviral role of IFN independent of NK cells is found in the LCMV system. The studies of Gresser and his colleagues (107) clearly show that treatment of mice with antibody to IFN dramatically increases LCMV synthesis in these mice. Our data show that injection of mice with anti-asialo GM1 totally depletes NK cell activity, while having no effect on either IFN levels or LCMV titers (II). These results show that the antiviral effect of IFN is independent of NK cells in the case of LCMV infection. The data in other viral systems is less clear, but the potent role of endogenous IFN against VV, MCMV, and HSV-1 in vivo (24,108,109) belies the fact that these viruses are relatively insensitive to IFN in vitro (24). This suggests that IFN may be
mediating antiviral effects via its action on host defense mechanisms, such as NK cells, which appear to have a potent antiviral effect against these 3 viruses (79, II, III, V).

The relative importance of and the interaction between IFN and NK cells has been studied in the MCMV system more extensively than in any other. Most laboratories (46, 98, 108, II, III) agree that NK cells may be important in the defense against MCMV, and we now have definitive evidence showing this (V). However, the relative importance of IFN and whether or not it has significant NK cell-independent antiviral effects against MCMV in vivo is controversial. Grundy-Chalmer et al. (108) showed a correlation between early IFN production and resistance to MCMV, but they did not rule out that the resistance was NK cell-mediated. Our data (II, III) show that NK cell depletion results in up to a 1000-fold increase in MCMV titers despite substantial rises in IFN titer 6-72 hours postinfection, thus showing that high levels of IFN in the absence of NK cells are not correlated with protection.

Chong et al. (109) attempted to determine whether or not IFN could mediate antiviral effects independently of NK cells. Injection of antibody to IFN caused NK cell-deficient beige mice to synthesize 3 times as much virus as compared to control beige mice, when assayed at 3 days postinfection, while having no effect on the NK cell activity on a per cell basis in these mice. However, these authors reported spleen necrosis and leukopenia caused by anti-IFN treatment of MCMV-infected mice, and it is therefore likely that anti-IFN-treated mice had fewer NK cells than control MCMV-infected
mice. Therefore the modest increase in MCMV titers caused by the anti-IFN could have been due to the effect of the antibody on NK cells rather than to the elimination of an NK cell-independent IFN-mediated antiviral effect. The beige mouse is an imperfect model for NK cell depletion, as these mice exhibit significant NK cell activity, especially during virus infection (46,II). A better model to study this question is that of an anti-asialo GMI-treated mouse, where total depletion of NK cell activity has been achieved, even in the face of a virus infection (II,III). It would be interesting to see what effect anti-IFN treatment has on MCMV replication in anti-asialo GMI-treated mice.

With regard to prophylactic treatment of mice with IFN before MCMV infection, Cruz et al. (110) showed that injection of suckling mice with IFN before MCMV challenge resulted in a modest (2-fold) reduction in salivary gland MCMV titers 12 days postinfection. More importantly, they noted that IFN pretreatment resulted in a marked inhibition of MCMV-induced necrosis in the liver, salivary glands, and brown fat. It is difficult to attribute this IFN-mediated protective effect strictly to reduction in virus titers, because the magnitude of the reduction was so small. In preliminary experiments we treated adult mice with the IFN inducer poly I:C 24 hours before challenge with MCMV. Three days later, these mice had much less severe liver injury than MCMV-infected control mice, as judged by gross examination, but the same amount of liver virus. Thus, IFN may be protecting liver cells and salivary gland cells from injury, either by direct effects on these cells (unrelated to inhibition of
virus synthesis), or inhibition of leukocyte infiltration, or both. I have already discussed the fact that IFN can protect normal cells from NK cell-mediated lysis (see previous section); perhaps this is the mechanism by which IFN may inhibit tissue injury. It therefore seems that IFN, while possibly having a slight NK cell-independent antiviral role during MCMV infection, may play more important roles by activating NK cells (whose antiviral effect against MCMV is orders of magnitude higher than any yet demonstrated for IFN alone) and by preventing tissue injury.

IFN may also have effects on the specific immune response during a virus infection. IFN may be required for the generation of CTL (72), and gamma IFN is produced by CTL upon recognition of appropriate targets (111). Our data show that virus-infected low-passage fibroblasts treated with IFN are 2 to 3 times more susceptible to virus-specific CTL-mediated lysis as compared to control virus-infected cells (IV-Fig.1). The increased sensitivity to lysis correlated with increased expression of surface H-2 antigens, but not viral antigens (IV-Figs.2 and 3). Our hypothesis is that IFN-induced increases in surface class I antigen expression (112,113) leads to increased association of these antigens with viral antigens, creating more structures which are recognizable by virus-specific CTL, thus increasing the chances that CTL will be bound to the targets, resulting in enhanced lysis of targets. That H-2 antigens are pivotal in this scheme is supported by the fact that IFN had no effect on H-2 expression (IV-Fig.3) or on susceptibility to lysis (IV-Fig.1) of cell lines already expressing
high levels of H-2 antigens.

These observations may be important with regard to the role of T cells in clearance of virus. Since MHC class I-restricted virus-specific T cells are known to eliminate virus in vivo (80), it is possible that IFN enhances this process by increasing the sensitivity of virus-infected cells to lysis by CTL. The source of the IFN could be virus-induced alpha or beta, or it may be gamma IFN produced by T cells upon recognition of a target (111). T cell-produced gamma IFN may locally enhance MHC expression in focal areas of infection, thereby increasing the sensitivity of T cell recognition of virus-infected tissue. Recent findings by Pfau and his associates (107) show that IFN treatment of mice infected intracranially with LCMV leads to increased mortality. Since death in this system has been shown to be caused by virus-specific T cell-dependent destruction of brain tissue (73), it is possible that IFN may be enhancing H-2 antigen expression on the surface of brain tissue, leading to more destruction of LCMV-infected cells by virus-specific T cells. This IFN-induced enhancement of MHC antigens on virus-infected cells may thus possibly augment T cell-dependent immunopathology as well as T cell-dependent clearance of virus. IFN may therefore facilitate antiviral cytotoxicity by selectively protecting uninfected targets from NK cells and by inducing H-2 expression, which could enhance surveillance by virus-specific T cells.
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