

ADOPTIVE TRANSFER STUDIES DEMONSTRATING THE  
ANTIVIRAL EFFECT OF NATURAL KILLER CELLS  
IN VIVO

BY JACK F. BUKOWSKI, JOHN F. WARNER,\* GUNTHER DENNERT,\* AND  
RAYMOND M. WELSH

*From the Departments of Pathology, and Molecular Genetics and Microbiology, University of  
Massachusetts Medical Center, Worcester, Massachusetts 01605; and the \*University of  
Southern California Comprehensive Cancer Center, Los Angeles, California 90033*

Natural killer (NK)<sup>1</sup> cells become activated (reviewed in 1) and proliferate (2, 3) during the early stages of virus infections, and they have been hypothesized to 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. Data in support of this hypothesis have been subject to other interpretations. The main approach has been to correlate susceptibility to virus infection with NK cell activity, which can be determined genetically or manipulated experimentally.

The best evidence, using this approach, has been obtained in the case of murine cytomegalovirus (MCMV) infection. Studies have shown that genetic resistance to MCMV correlates with NK cell activity in mice (5, 6). Homozygous beige mice, which have a defect in NK cell-mediated cytotoxicity, are highly susceptible to MCMV, and 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 (10, 11) we have shown that antiserum to asialo GM<sub>1</sub>, a reagent which, rather selectively, eliminates NK cell activity in mice, greatly enhances MCMV growth and pathogenesis. Biological response modifiers that activate NK cells in vivo, in the absence of interferon (IFN), render mice more resistant to MCMV; this resistance is abrogated by antiserum to asialo GM<sub>1</sub> (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 that affects functions other than NK cells (13), and asialo GM<sub>1</sub> is a common molecule found on several cell types (14, 15). To more

This research was supported by U. S. Public Health Research Grants AI-17672 and CA 34461 (to R. M. Welsh), CA 37706, CA39501, and American Cancer Society Grant IM 284 to G. Dennert.

<sup>1</sup> *Abbreviations used in this paper:* C', complement; FBS, fetal bovine serum; IFN, interferon; IL-2, interleukin 2; LCMV, lymphocytic choriomeningitis virus; mAb, monoclonal antibody; MCMV, murine cytomegalovirus; MEF, mouse embryo fibroblast; MEM, minimum essential medium; NK, natural killer; PFU, plaque-forming units; VSV, vesicular stomatitis virus.

definitively assess the role of NK cells in virus infections, we have developed cellular adoptive transfer methods that 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 GM<sub>1</sub> (10). We report here that the transfer of only those populations of cells containing NK cell activity can protect suckling mice against MCMV, but leave them unprotected from LCMV. Furthermore, adoptive transfer of cloned NK cells, but not T cells, provides 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) viral infection.

### Materials and Methods

*Animals.* C57BL/6 and BALB/c mice were purchased from The Jackson Laboratories, Bar Harbor, ME, and bred in our facility. SWR/J athymic nude mice were a gift from Dr. Aldo Rossini, University of Massachusetts Medical Center. Donor mice for adoptive transfer studies were 4–8 wk 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 Laboratories, 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 Laboratories) with the same additives as listed above. Baby hamster kidney 21/13S cells were maintained in Dulbecco's modified Eagle's medium (Gibco Laboratories) 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 2–3 wk previously with 10<sup>4</sup> 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 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 μCi [<sup>51</sup>Cr]sodium chromate (New England Nuclear, Boston, MA) for 1 h at 37°C, were washed and mixed with various numbers of effector cells in round-bottomed microtiter wells at 10<sup>4</sup> 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 4–16 h at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> 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, Inc., Palo Alto, CA). Data are expressed as percent specific release: 100 × [(cpm experimental – cpm spontaneous)/(cpm maximum – cpm spontaneous)]. Spontaneous release of radioactivity was 10–20%. Standard deviations of quadruplicate replica samples were <10% of 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 titers from four separately titrated individual animals, plus or minus 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 a Student's *t* test.

*Antisera.* Rabbit antiserum against asialo GM<sub>1</sub> 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 GM<sub>1</sub> was diluted 1:10 in RPMI medium and given intravenously in a volume of 0.2 ml, 4–6 h before challenge with virus. In adoptive transfer experiments, donor spleen cells from control mice treated with anti-asialo GM<sub>1</sub> were used 18–24 h after antibody treatment. Monoclonal anti-Thy-1.2 antibody (mAb) was provided by Dr. Edward Clark, University of Washington, Seattle, WA and used at a final dilution of 1:900. Anti-Ly-5.1 (clone M1/89.18) and anti-Ia (clone M5/114) mAb, gifts from Dr. Eric Martz, University of Massachusetts, Amherst, MA (21), were used at a 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,  $2.5\text{--}4.0 \times 10^8$  spleen leukocytes were suspended in 2.5 ml RPMI containing antibody, and incubated for 20–30 min at room temperature with occasional agitation. 250  $\mu$ l of guinea pig serum (a source of complement; C') was 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.* NK1B6B10, a clone of interleukin 2 (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 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM MEM nonessential amino acids (Gibco Laboratories),  $5 \times 10^{-5}$  M 2-mercaptoethanol, 50 U/ml penicillin and streptomycin, 100  $\mu$ g/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-cm<sup>2</sup> flasks were washed, and adherent cells were removed with trypsin-EDTA (Gibco Laboratories) and diluted 1:4 in growth medium. This suspension was then incubated at 37°C for 1 h in a conical test tube, with occasional agitation. Cells were then counted, pelleted at 200 *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 cell 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 d old. Before use in experiments, mice from several litters were pooled and randomly reassigned to lactating mothers. Groups of four to nine mice were given an injection of cloned NK cells or mouse spleen cells treated in various ways. We delivered 0.1 ml 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 in a volume of 0.1 ml i.p. Mice were monitored daily for survival. Some were killed 3 d later, and their spleens removed and used for virus titration. In some experiments, using the cloned NK cells (23), 4-wk-old C57BL/6 mice were given four weekly doses of 200 rad  $\gamma$  radiation from a <sup>60</sup>Co source. 5 wk after the last dose, the mice were given 10  $\mu$ l anti-asialo GM<sub>1</sub> intravenously, followed 2 d later by  $4 \times 10^6$  cloned NK cells, half given intraperitoneally, the other half given intravenously. 6 d later, intraperitoneal and intravenous injections were again administered, each consisting of  $10^6$  cloned NK cells. 10 d after the last injection, groups of five mice were challenged with an intraperitoneal injection of  $10^4$  PFU of MCMV. Controls consisted of irradiated, anti-asialo GM<sub>1</sub>-treated mice that had not received injections of NK cells. 3 d later, the mice were sacrificed, and their spleens and livers were titrated for virus.

*Interferon Assay.* Blood was collected in heparinized Natelson tubes. Mice were anesthetized with ether before being bled from the retroorbital sinus. Plasma was obtained by centrifugation. Plasma were titrated by twofold serial dilution in a 96-well, flat-bottomed microtiter plate to which L-929 cells were added at  $3 \times 10^4$  cells/well. 18–24 h later, the wells were challenged with 100 TCID<sub>50</sub> U of VSV. IFN titers were expressed as the log<sub>2</sub> of the highest reciprocal dilution that resulted in a 50% reduction in cytopathic effect.

Results are expressed as the geometric mean titers of separate animals titrated for IFN individually  $\pm$  SEM.

*Centrifugal Elutriation.* Size separation of cells was accomplished using a Beckman JE-6B centrifuge (2).  $\sim 10^9$  spleen leukocytes were treated with deoxyribonuclease (Sigma Chemical Co.) to prevent clumping. These cells were then loaded into a rotor that was spinning at 3,200 rpm at 5°C. The rate of flow of elution medium (Hanks' balanced salt solution, 1.5% calf serum) was 15, 22, 28, and 46 ml/min, corresponding to fractions 1-4. Cell recovery was  $\sim 65\%$ . 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 further separated on discontinuous Percoll gradients according to the method of Kumagai et al. (25), with some modifications. One part 10 $\times$  phosphate-buffered saline and one part FBS were added to eight parts Percoll to obtain 80% Percoll. This was further diluted using RPMI 1640 containing 10% FBS.  $6-7 \times 10^7$  cells were suspended in 3 ml 64% Percoll and pipetted into a 15 ml conical test tube. 3 ml of 59, 50, and 37% Percoll were sequentially layered on top of the cell suspension. Centrifugation was at 300 g and 20°C for 45 min. 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.  $6 \times 10^7$  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, since 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). Selgrade and Osborn (17) have shown that adult spleen leukocytes can protect suckling mice against lethal MCMV infection. Fig. 1A 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. Fig. 1B shows that  $5 \times 10^7$  spleen leukocytes from 5-wk-old donors were significantly more effective than those from 17-d-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  $\sim 98\%$  lymphocytes, and  $<1\%$  of the cells were capable of phagocytizing yeast particles, indicating minimal contamination with macrophages. Fig. 1C 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-d-old mice.

*T Cell Depletion Does Not Affect Protective Capacity.* Spleen leukocytes were depleted of T cells by treatment with anti-Thy-1.2 mAb and C', then adoptively transferred into recipient suckling mice. This treatment kills  $\sim 35\%$  of the cells, eliminates LCMV-specific cytotoxic T cell activity of sensitized spleen cells, but has little effect on NK cell activity (19). Fig. 1D 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

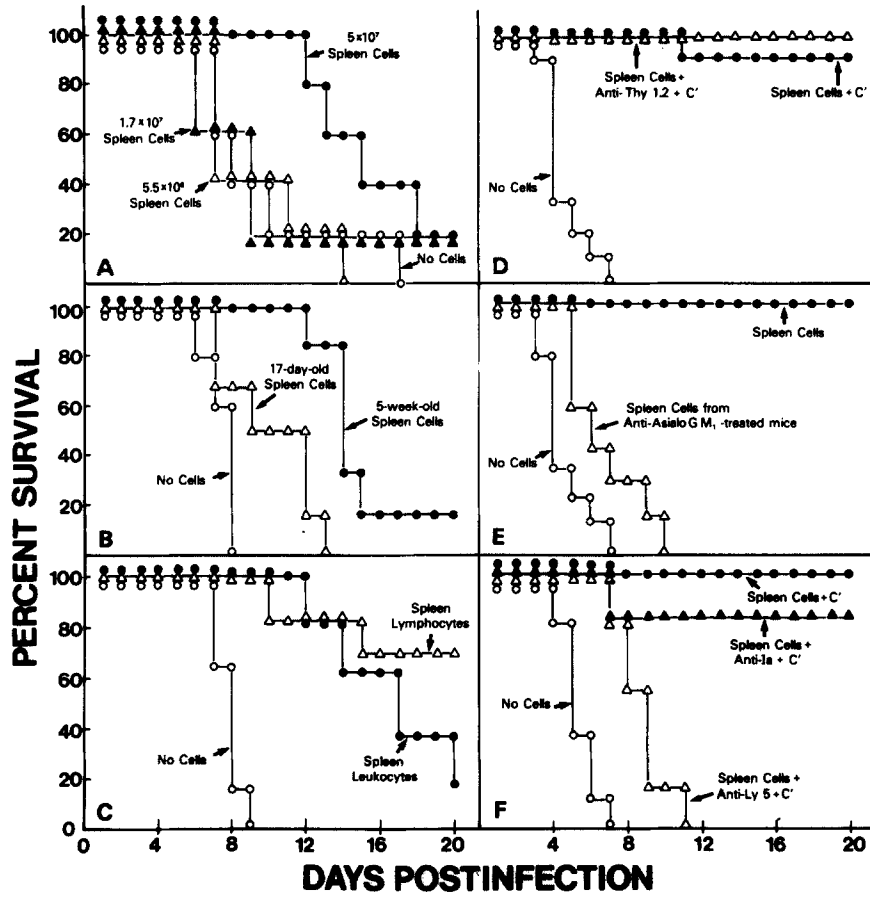


FIGURE 1. Effect of adoptive transfer of various spleen cell populations on survival of MCMV-infected suckling mice. Suckling mice 3–5 d old were (unless otherwise indicated) given  $5 \times 10^7$  spleen cells (which were left untreated or fractionated as described in Materials and Methods), or medium alone. This intraperitoneal injection was followed 24 h later by  $6 \times 10^5$  PFU of MCMV, also given intraperitoneally. Mice were monitored for survival for 20 d. (A) Various numbers of adult spleen leukocytes were transferred. Five mice per group. (B) Spleen leukocytes from suckling and weanling mice were used as donor cells. Five to six mice per group. (C) Spleen leukocytes or size-separated lymphocytes were used as donor cells. Six to seven mice per group. (D) Spleen leukocytes treated with C' or anti-Thy-1.2 and C' were used as donor cells. Seven mice per group. (E) Spleen leukocytes from control or anti-asialo GM<sub>1</sub>-treated mice were used as donor cells. Seven to nine mice per group. (F) Spleen leukocytes treated with C', anti-Ly-5 and C', or anti-Ia and C' were used as donor cells. Six mice per group.

cells. Another line of evidence indicating that protection can occur without T cells is shown in Fig. 2. Athymic nude mice were either left untreated or selectively depleted of NK cell activity by injection with anti-asialo GM<sub>1</sub>. 4–6 h later, the mice were challenged with MCMV. The data show that MCMV-infected NK cell-depleted mice had five to six times more liver virus and two to three times more spleen virus than control MCMV-infected mice. This provides evidence that T cells are not required for the anti-asialo GM<sub>1</sub>-mediated exacerbation of MCMV infection.

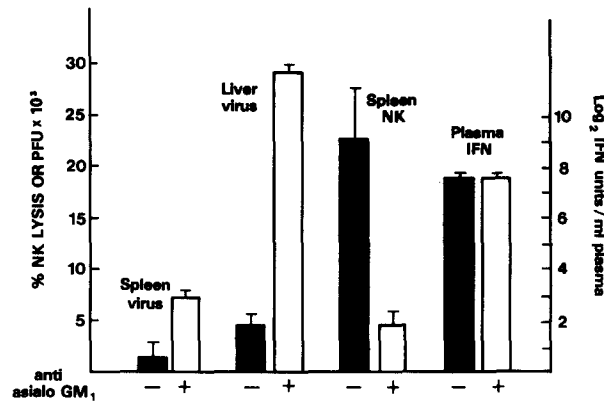


FIGURE 2. NK cell depletion lowers resistance to MCMV in nude mice. SWR/J athymic nude mice were left untreated, or given 20  $\mu$ l anti-asialo GM<sub>1</sub> intravenously. 4–6 h later, these mice were challenged with  $5 \times 10^5$  PFU of MCMV intraperitoneally. 3 d later, the mice were killed, and their organs were titrated for MCMV, their spleens assayed for NK cell activity, and their plasma titrated for IFN. Four mice per group.

*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 GM<sub>1</sub> 18–24 h earlier) were adoptively transferred into suckling mice. This treatment reduced NK cell activity from 21 to ~2.7%. Fig. 1E shows that the cells from mice depleted of NK activity by anti-asialo GM<sub>1</sub> failed to prevent death of mice infected with MCMV, whereas control leukocytes totally prevented death. Treatment of leukocytes with anti-Ly-5 mAb and C' reduced NK cell activity from 20 to 8.6%, and eliminated the protection. But treatment with anti-Ia mAb 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. 1F). 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 Cell-enriched Leukocytes.* Since previous studies (11) showed that increased survival correlates with lower spleen MCMV titers, we titrated the spleens of MCMV-infected suckling mice 3 d postinfection. The data in Table I, A–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 data in Fig. 1, which show that cells depleted of B cells and macrophages can protect against MCMV (Table IA). Kumagai et al. (25) have shown that large granular lymphocytes containing NK activity can be purified on gradients of Percoll. With a similar technique, nylon wool-passed leukocytes were subfractionated in Percoll gradients to enrich for, or to deplete NK cells (Table IB). The experiment depicted in Fig. 1A 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 IB shows that  $1.7 \times 10^7$  control spleen cells resulted in only a three- to fivefold reduction in MCMV titers in the recipient spleens, but that the same number of cells, enriched threefold for NK cell activity (in low density fractions after Percoll purification) resulted in a (~30-fold) decrease in

TABLE I  
Reduction of MCMV Titers by Adoptive Transfer of Cell Populations Containing NK Cell Activity\*

Exp.	E/T <sup>‡</sup>	Percent NK cell lysis <sup>§</sup> by donor cells	Number of cells used, and treatment	log <sub>10</sub> PFU of MCMV per spleen
A		ND	Medium alone	4.1 ± 0.1
			5 × 10 <sup>7</sup> Control spleen	2.5 ± 0.2 <sup>†</sup>
			5 × 10 <sup>7</sup> Nylon wool-passed spleen	2.3 ± 0.2 <sup>‡</sup>
B	6	0.2	Medium alone	2.9 ± 0.1
			1.7 × 10 <sup>7</sup> Percoll, high density	3.1 ± 0.1
			1.7 × 10 <sup>7</sup> Percoll, low density	1.5 ± 0.2 <sup>‡</sup>
			1.7 × 10 <sup>7</sup> Control spleen	2.4 ± 0.1
		14.0	5.0 × 10 <sup>7</sup> Control spleen	1.4 ± 0.2
C	25	29.0	Medium alone	4.1 ± 0.1
			5 × 10 <sup>7</sup> Control spleen	2.7 ± 0.1
		24.0	5 × 10 <sup>7</sup> Control spleen + C'	2.8 ± 0.1
		8.5	5 × 10 <sup>7</sup> Control spleen + anti-NK 1.2 ± C'	3.7 ± 0.1**
D	50	23.	Medium alone	4.6 ± 0.1
			5 × 10 <sup>7</sup> Control spleen	3.2 ± 0.1
		3.3	5 × 10 <sup>7</sup> Anti-AGM <sub>1</sub> spleen	4.1 ± 0.1 <sup>‡‡</sup>
		ND	5 × 10 <sup>5</sup> Cloned NK cells	1.9 ± 0.1
E	20	40.0	Medium alone	4.5 ± 0.1
			1.2	5 × 10 <sup>5</sup> Cloned NK cells
			5 × 10 <sup>5</sup> Cloned T cells	4.4 ± 0.1

\* Groups of four suckling mice were given intraperitoneal injections of cells treated as indicated above, or RPMI 1640 medium. The next day, they were challenged with 6 × 10<sup>5</sup> PFU of MCMV. 3 d later the mice were killed and their spleens titrated for MCMV. (Exp. A) Donor spleen cells were either left untreated or passed over nylon wool columns, as described in Materials and Methods. (B) Donor spleen cells were left untreated, or fractionated by nylon wool passage and Percoll density gradient centrifugation, as described in Materials and Methods. (C) Donor spleen cells were left untreated, treated with C', or treated with anti-NK 1.2 and C', as described in Materials and Methods. (D) Donor spleen cells were from control mice or mice treated 24 h earlier with anti-asialo GM<sub>1</sub>. Donor NK cells were the cloned NK1B6B10 line. (E) Donor NK cells were the cloned NK1B6B10 line, and T cells were CTLL-2 clone 15 H.

<sup>‡</sup> Effector/target ratio.

<sup>§</sup> Percent NK cell lysis refers to percent specific release against YAC-1 targets at a given E/T ratio, as described in Materials and Methods.

<sup>†</sup> P < 0.001 as compared with medium.

<sup>‡</sup> P < 0.01 as compared with 1.7 × 10<sup>7</sup> control spleen cells.

\*\* P < 0.01 as compared with control spleen + C'.

<sup>‡‡</sup> P < 0.01 as compared with control spleen.

<sup>§§</sup> P < 0.001 as compared with cloned T cells.

MCMV titers. Mice receiving an equal number of Percoll-separated high density cells devoid of NK cell activity were not protected at all. Thus, nylon wool-passed, Percoll gradient-fractionated spleen cells enriched for NK cell activity have an enhanced capacity to reduce virus titers, as compared with control spleen leukocytes, or those devoid of NK cell activity.

*Failure of NK Cell-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 IC) or spleen cells from anti-asialo GM<sub>1</sub>-treated mice (Table ID) resulted in recipient spleen MCMV titers that were nearly 10 times 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 I, D and E, show that these cells were extremely effective in reducing MCMV titers, as mice receiving the cloned NK cells had ~500-fold less MCMV in their spleens than did mice receiving an IL-2-dependent T cell clone (CTL-2 clone 15 H, also derived from C57BL/6 mice; 24) or no cells at all. On a cell/cell basis, the NK cell clone was >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

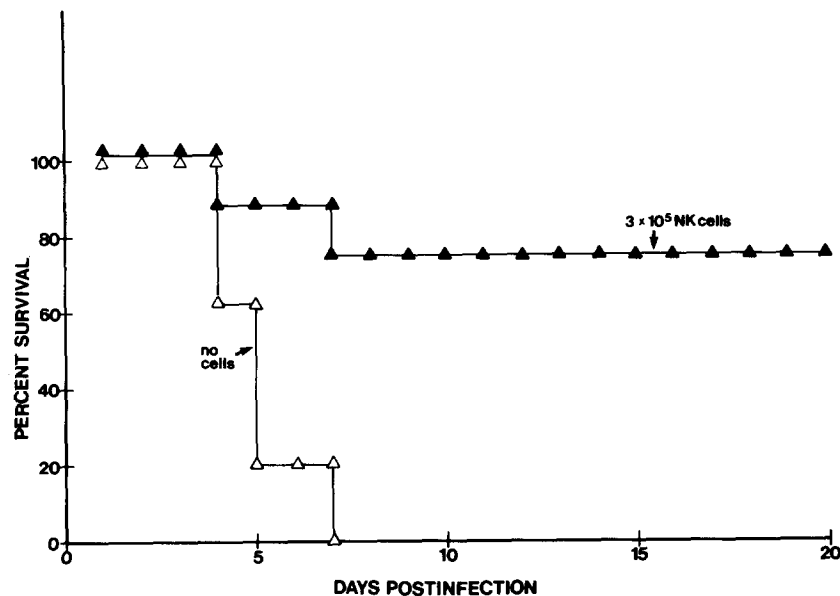


FIGURE 3. Cloned NK cells enhance survival of MCMV-infected suckling mice. Cloned NK cells were adoptively transferred into recipient suckling mice and then challenged 24 h later with  $6 \times 10^5$  PFU of MCMV. Control mice received no cells. Mice were observed 20 d for mortality. Eight mice per group.



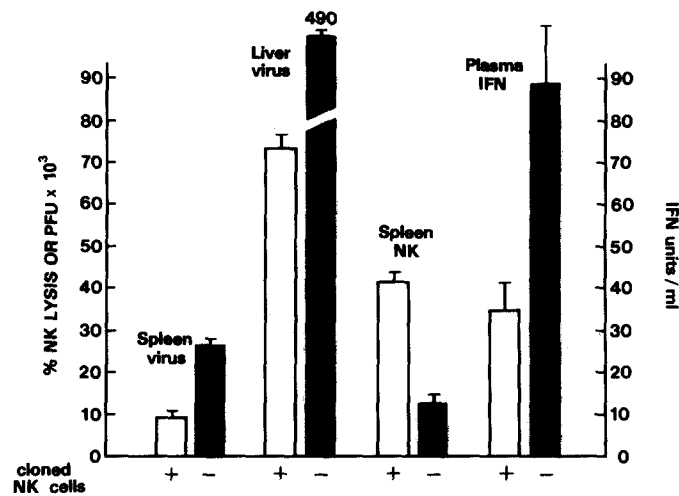


FIGURE 4. Cloned NK cells inhibit MCMV replication in adult mice. Adult C57BL/6 mice were irradiated and left untreated or injected with cloned NK cells, as described in Materials and Methods. These mice were then challenged with  $10^4$  PFU of MCMV intraperitoneally, and killed 3 d later. Spleens and livers were titrated for MCMV, spleens were assayed for NK cell activity, and plasma was titrated for IFN. Five mice per group.

TABLE II  
*Adoptive Transfer of Cell Populations Containing NK Cell Activity  
Has No Effect on LCMV Titers\**

Exp.	Number of cells used, and treatment	$\log_{10}$ PFU of LCMV per spleen
A	Medium alone	$4.7 \pm 0.1$
	$5 \times 10^7$ Control spleen	$4.4 \pm 0.1^\ddagger$
B	Medium alone	$4.7 \pm 0.1$
	$5 \times 10^5$ Cloned NK cells	$4.7 \pm 0.2^\ddagger$

\* Groups of four suckling mice were given intraperitoneal injections of control spleen cells, the NK cell clone NK1B6B10, or RPMI medium. The next day, they were challenged with  $8 \times 10^3$  PFU of LCMV. 3 d later, the mice were sacrificed and their spleens were titrated for LCMV.

$^\ddagger$  Not significantly different from medium.

of MCMV-infected mice, since 75% of mice receiving only  $3 \times 10^5$  NK cells survived, compared with none of the control mice (Fig. 3).

To test whether the NK cell clone could protect adult mice from MCMV, 6 wk-old mice were irradiated four times with 200 rad at weekly intervals to deplete NK cell activity, then given injections of the cloned NK cells. The reconstituted recipients had elevated spleen NK cell activity and reduced virus titers in their spleens and livers (Fig. 4). 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 in suckling mice.

*Adoptive Transfer of NK Cells Has No Effect on LCMV Titers.* Previous evidence (10, 16) had 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 challenged them the next day with LCMV. Table II, *A* and *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. The phenotype of the protective cell population is that of a nylon wool-nonadherent, asialo  $GM_1^+$ , NK 1.2<sup>+</sup>, Ly-5<sup>+</sup>, Thy-1<sup>-</sup>, Ia<sup>-</sup>, 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 2–3 wk, it is possible that during that 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 3 d postinfection (data not shown). Previous studies (11) with anti-asialo  $GM_1$  suggested that NK cells could mediate their antiviral effects up to 3 d postinfection, but not at 6–9 d. The present experiments, involving titration of MCMV in the spleen 3 d postinfection, confirm this result and indicate that it is not due to differentiation and sensitization of T cells over the long time period. T cells are reported to mediate antiviral effects 6–30 d postinfection (27). Further arguments against a role for T cells early in infection include the fact that NK cell depletion lowers the resistance of athymic nude mice to MCMV infection (Fig. 2), 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. 1*D*). Whereas some NK cells express some Thy-1 antigen (26), the concentrations of antiserum and C' used here deplete cytotoxic T lymphocyte 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. Whether unstimulated macrophages could provide resistance to MCMV remains at issue, 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 the transfer of resistance. Antibodies that leave macrophage function intact, such as anti-asialo  $GM_1$ , 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 d postinfection in adult mice, and 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 indicate that  $5 \times 10^7$  adult spleen cells could prolong survival of MCMV-infected mice, but that threefold fewer cells could not. If the protection were mediated by NK cells, then threefold fewer spleen cells with three times as much NK cell activity should protect. This prediction was supported by our data;  $1.7 \times 10^7$  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 threefold for NK cell activity, significantly reduced MCMV titers.

Previous work (29) 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. These cells have the phenotype: asialo GM<sub>1</sub><sup>+</sup>, NK 1.2<sup>+</sup>, Thy-1<sup>+</sup>, Ly-1<sup>-</sup>, Ly-2<sup>-</sup>, and are capable of mediating *in vitro* lysis against NK cell-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 VSV (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 our recently obtained evidence<sup>2</sup> 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

We carried out adoptive transfer studies to determine the role of natural killer (NK) cells in resistance to murine cytomegalovirus (MCMV) and lymphocytic choriomeningitis virus (LCMV). We transferred leukocytes from adult mice into suckling mice 1 d before injecting them with virus. Resistance was measured by enhancement of survival and reduction of virus multiplication in the spleens of recipient mice. The phenotype of the cell population capable of mediating

<sup>2</sup> Bukowski, J. F., and R. M. Welsh. 1984. Susceptibility of virus-infected targets to natural killer cell-mediated lysis *in vitro* correlates with natural killer cell-mediated antiviral effects *in vivo*. Submitted for publication.

resistance to MCMV was that of a nylon wool–nonadherent, asialo GM<sub>1</sub><sup>+</sup>, NK 1.2<sup>+</sup>, Ly-5<sup>+</sup>, Thy-1<sup>-</sup>, Ia<sup>-</sup>, 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<sub>1</sub>, 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 can protect mice from MCMV but not from LCMV.

*Received for publication 16 August 1984 and in revised form 9 October 1984.*

### References

1. Welsh, R. M. 1978. Mouse natural killer cells: induction, specificity, and function. *J. Immunol.* 121:1631.
2. Biron, C. A., and R. M. Welsh. 1982. Blastogenesis of natural killer cells during viral infection *in vivo*. *J. Immunol.* 129:2788.
3. Biron, C. A., L. R. Turgiss, and R. M. Welsh. 1983. Increase in NK cell number and turnover rate during acute viral infection. *J. Immunol.* 131:1539.
4. Welsh, R. M. 1981. Natural cell-mediated immunity during viral infections. *Curr. Top. Microbiol. Immunol.* 92:83.
5. Bancroft, G. J., G. R. Shellam, and J. E. Chalmer. 1981. Genetic influences on the augmentation of natural killer (NK) cells during murine cytomegalovirus infection: correlation with patterns of resistance. *J. Immunol.* 126:988.
6. Shellam, G. R., J. E. Allen, J. M. Papadimitriou, and G. J. Bancroft. 1981. Increased susceptibility to cytomegalovirus infection in beige mutant mice. *Proc. Natl. Acad. Sci. USA.* 78:5104.
7. Boos, J., and E. F. Wheelock. 1971. Correlation of survival from MCMV infection with spleen cell responsiveness to concanavalin A. *Proc. Soc. Exp. Biol. Med.* 149:443.
8. Henson, D., R. D. Smith, and J. Gehrke. 1966. Non-fatal mouse cytomegalovirus hepatitis. Combined morphologic, virologic, and immunologic observations. *Am. J. Pathol.* 49:871.
9. Quinnan, G. V., Jr., J. E. Manischewitz, and N. Kirmani. 1982. Involvement of natural killer cells in the pathogenesis of murine cytomegalovirus interstitial pneumonitis and the immune response to infection. *J. Gen. Virol.* 58:173.
10. Bukowski, J. F., B. A. Woda, S. Habu, K. Okumura, and R. M. Welsh. 1983. Natural killer cell depletion enhances virus synthesis and virus-induced hepatitis *in vivo*. *J. Immunol.* 131:1531.
11. Bukowski, J. F., B. A. Woda, and R. M. Welsh. 1984. Pathogenesis of murine cytomegalovirus infection in natural killer cell-depleted mice. *J. Virol.* 52:119.
12. Ebihara, K., and Y. Minamishima. 1984. Protective effect of biological response modifiers on murine cytomegalovirus infection. *J. Virol.* 51:117.
13. Vassalli, J.-D., A. Granelli-Piperno, C. Griscelli, and E. Reich. 1978. Specific protease deficiency in polymorphonuclear leukocytes of Chédiak-Hagashi syndrome and beige mice. *J. Exp. Med.* 147:1285.
14. Kasai, M., M. Iwamori, Y. Nagai, K. Okumura, and T. Tada. 1980. A glycolipid on the surface of mouse natural killer cells. *Eur. J. Immunol.* 10:175.
15. Habu, S., H. Fukui, K. Shimamura, M. Kasai, Y. Nagai, K. Okumura, and N. Tamaoki. 1981. *In vivo* effects of anti-asialo GM<sub>1</sub>. Reduction of NK activity and enhancement of transplanted tumor growth in nude mice. *J. Immunol.* 127:34.

16. Welsh, R. M., and R. W. Kiessling. 1980. Natural killer cell response to lymphocytic choriomeningitis virus in beige mice. *Scand. J. Immunol.* 11:367.
17. Selgrade, M. K., and J. E. Osborn. 1974. Role of macrophages in resistance to murine cytomegalovirus. *Infect. Immun.* 10:1383.
18. Nedrud, J. G., A. M. Collier, and J. S. Pagano. 1979. Cellular basis of susceptibility to murine cytomegalovirus: evidence from tracheal organ culture. *J. Gen. Virol.* 45:737.
19. Kiessling, R., E. Eriksson, L. A. Hallenbeck, and R. M. Welsh. 1980. A comparative analysis of the cell surface properties of activated vs. endogenous mouse natural killer cells. *J. Immunol.* 125:1551.
20. Kawase, I., D. L. Urdal, C. G. Brooks, and C. S. Henney. 1982. Selective depletion of NK cell activity *in vivo* and its effect on the growth of NK sensitive and NK resistant tumor cell variants. *Int. J. Cancer.* 29:567.
21. Davignon, D., E. Martz, T. Reynolds, K. Kurzinger, and T. A. Springer. 1981. Lymphocyte function-associated antigen 1 (LFA-1): a surface antigen distinct from Ly-2, 3 that participates in T lymphocyte-mediated killing. *Proc. Natl. Acad. Sci. USA.* 78:4535.
22. Burton, R. C. 1980. Alloantigens selectively reactive with NK cells: characterization and use in defining NK cell classes. *In: Natural Cell Mediated Immunity Against Tumors.* R. B. Herberman, editor. Academic Press, Inc., New York. 19-23.
23. Dennert, G. 1980. Cloned lines of natural killer cells. *Nature (Lond.)* 237:47.
24. Baker, P. E., S. Gillis, and K. A. Smith. 1979. Monoclonal cytotoxic T-cell lines. *J. Exp. Med.* 149:273.
25. Kumagai, K., K. Itoh, R. Suzuki, S. Hinuma, and F. Saitoh. 1982. Studies of murine large granular lymphocytes. I. Identification as effector cells in NK and K cytotoxicities. *J. Immunol.* 129:388.
26. Welsh, R. M. 1984. Interferon and natural killer cells. *CRC Crit. Rev. Immunol.* 5:55.
27. Ho, M. 1980. Role of specific cytotoxic lymphocytes in cellular immunity against murine cytomegalovirus. *Infect. Immun.* 27:767.
28. Araullo-Cruz, T., M. Ho., and J. A. Armstrong. 1978. Protective effect of early serum from mice after cytomegalovirus infection. *Infect. Immun.* 21:840.
29. Warner, J. F., and G. Dennert. 1982. Effects of a cloned cell line with NK activity on bone marrow transplants, tumour development, and metastases *in vivo*. *Nature (Lond.)* 300:31.
30. Lopez, C. 1981. Resistance to herpes simplex virus type I (HSV-1). *Curr. Top. Microbiol. Immunol.* 92:15.
31. Tardieu, M., C. Hery, and J. M. Dupuy. 1980. Neonatal susceptibility to MHV infection in mice. II. Role of natural effector marrow cells in transfer of resistance. *J. Immunol.* 124:418.
32. Kumar, V., and M. Bennett. 1981. Genetic resistance to Friend virus-induced erythroleukemia and immunosuppression. *Curr. Top. Microbiol. Immunol.*, 92:65.
33. Minato, N., B. R. Bloom, C. Jones, J. Holland, and L. M. Reid. 1979. Mechanism of rejection of virus persistently infected tumor cells by athymic nude mice. *J. Exp. Med.* 149:1117.
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.