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Blastogenesis of Large Granular Lymphocytes in Nonlymphoid Organs

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High numbers of large granular lymphocytes (LGL) accumulate in the livers and peritoneal cavities of mice during the course of viral infection. Accumulation of natural killer (NK) cells at day 3 postinfection (p.i.) was shown to be radiation-sensitive, implying that proliferation was required for this response. Accumulation occurred in splenectomized mice, indicating that the spleen, known to be an organ for mature NK cell proliferation, was not the major source for liver and peritoneal NK/LGL.

Significant percentages (> 25%) of the LGL found in the liver and peritoneal cavity following viral infection or interferon induction with poly-inosinic:poly-cytidylic acid were defined morphologically as blasts (large cells with prominent nucleoli and intensely basophilic cytoplasms containing azurophilic granules). Most blast LGL at day 3 p.i. were sensitive to administration of anti-asialo GM_1 serum in vivo, were Lyt-2^-, and were enriched in populations that lysed NK cell-sensitive targets in vitro, indicating that these were NK/LGL. At day 3 p.i., leukocytes from the liver and peritoneal cavity incorporated ^{3}H-thymidine and bound to and killed NK cell-sensitive targets in single-cell cytotoxicity assays. These data suggest that NK/LGL undergo at least one round of division in the liver and peritoneal cavity during viral infection.

In contrast, blast LGL at day 7 p.i. were resistant to in vivo treatments with anti-asialo GM_1 serum, were Lyt-2^+, and were enriched in populations of cells that killed virus-infected histocompatible targets, indicating that they were cytotoxic T lymphocytes (CTL). These results suggest that both NK/LGL and CTL/LGL are capable of blastogenesis and presumed proliferation at sites of virus infection, providing a means for the in situ augmentation of a host's cell-mediated antiviral defenses.

Key words: cytotoxic T lymphocyte, natural killer cell, virus infection

INTRODUCTION

Both natural killer (NK) cells [42,26] and cytotoxic T lymphocytes (CTL) [5] are large granular lymphocytes (LGL) that infiltrate infected tissue during virus infection [33,36]. NK/LGL provide natural resistance to certain viruses early in infection [10,11], whereas virus-specific CTL/LGL are generated later in infection and are responsible for virus clearance and recovery of the host [48]. The mechanisms by which LGL accumulate in infected tissue are not understood.

NK cell precursors are found predominantly in the bone marrow, and the bone marrow microenvironment is essential for the maturation of NK cells and the maintenance of NK cell activity [16,17,27]. Neutrophil, macrophage, and B- and T-lymphocyte precursors also arise from the bone marrow, but with different fates. Neutrophils are released from the bone marrow and migrate into tissue in a mature, end-stage form, incapable of cell division [4,30]. Immature monocytes are released from the marrow, but they differentiate into macrophages and a variety of histiocytes after infiltration of tissue. There they are capable of limited amounts of cell division [13,22]. Early in life, immature bone marrow-derived B cells [reviewed in 25] and T cells [reviewed in 40] seed the lymph nodes, spleen, and thymus. Here they undergo high levels of antigen-independent (thymus) and antigen-dependent (lymph node, spleen) proliferation and maturation in germinal centers. Mature, functional NK cells exist in the normal murine liver [33,48], but whether significant levels of NK cell maturation and proliferation can occur outside of lymphoid and hematogenous tissues is less clearly understood.

Mature NK cells in the spleen undergo blastogenesis and proliferation during the first few days of viral infection, or following injection of mice with interferons (IFN)

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or with IFN inducers [6,8]. Under these conditions, the peripheral blood, bone marrow, and lymph nodes appear to contain relatively few functional proliferating NK cells [45], but the number of NK cells in virus-infected organs such as the liver and peritoneal cavity increases 5- to 50-fold [33,36]. These massive increases of NK/LGL in infected organs could be due to infiltration with precursors of NK cells from the bone marrow, to infiltration with mature NK cells after proliferation in the spleen, and/or to in situ proliferation of NK cells within the infected organs. In this paper we examine two nonlymphoid compartments of the murine host, the liver and the peritoneal cavity, for the presence of blast LGL. We find that LGL of both NK cell and CTL phenotypes undergo blastogenesis and presumed proliferation within these sites during the course of virus infection.

MATERIALS AND METHODS

Mice

Mice were used between the ages of 6 and 12 weeks. C3H/St male mice were purchased from West Seneca Laboratories (West Seneca, NY). C57BL/6J mice and C57BL/6J mice carrying the beige (bg) mutation were purchased from Jackson Laboratories (Bar Harbor, ME). Athymic nude (nu/nu) mice (BALB/c background) were bred in our facilities.

Viruses

The hepatotropic WE strain [46] of lymphocytic choriomeningitis virus (LCMV-WE), the nonhepatotropic Armstrong strain of lymphocytic choriomeningitis virus (LCMV-ARM) [44], and Pichinde virus, an arenavirus related to LCMV [9], were grown in L-929 cells [47]. Mouse hepatitis virus, A59 strain (MHV) [40], was a liver-grown stock propagated in vivo, and murine cytomegalovirus, Smith strain (MCMV) [38], was a salivary gland-grown stock. Mice were infected intraperitoneally (i.p) with 10^5 plaque-forming units (PFU) of LCMV-WE or 8×10^4 PFU of LCMV-ARM in a volume of 0.1 ml. MHV (3×10^5 PFU) was injected intravenously (i.v.) or i.p., and MCMV (1×10^4 PFU) was injected i.p. Sucrose gradient-purified vaccinia virus, strain IHDJ [35], was injected i.p. (1×10^7 PFU).

Cells

YAC-1 cells, derived from a Moloney leukemia virus-induced lymphoma in A/Sn mice [14,24], and K-562 human chronic myelogenous leukemia cells [29] were maintained in RPMI 1640 medium (GIBCO, Grand Island, NY) supplemented with antibiotics, glutamine, and 10% heat-inactivated fetal bovine serum (M.A., Bioproducts, Walkerville, MD). L-929 cells, a continuous cell line from C3H mice, and the MC57G cell line from C57BL/6 mice, were maintained in minimal essential medium (MEM, GIBCO) with the same additives listed above. In some cytotoxicity assays, target cells were infected with virus (0.1 PFU/cell) 48 hrs prior to use.

Reagents

Poly-inosinic:poly-cytidylic acid (poly I:C; Sigma Chemical Co., St. Louis, MO) was injected i.p. (100 μg) 2 days prior to use of mice.

Antisera

Rabbit anti-ganglio-tetraosylceramide (anti-asialo GM_1; Wako Chemicals, Dallas, TX) was used to selectively deplete NK cell activity in vivo [15]. Anti-asialo GM_1 was diluted 1:10 with RPMI medium, and 0.2 ml was injected i.v.

Preparation of Leukocytes From the Liver and Peritoneal Cavity

Peritoneal exudate cells (PEC) were leukocytes obtained from the peritoneal cavities of mice either decapitated or anesthetized with sodium pentobarbital (1–2 mg, i.p.) by peritoneal lavage with 4 ml serum-free medium.

Leukocytes were isolated from livers of mice as described previously [33]. Briefly, following decapitation and exsanguination or injection of sodium pentobarbital (1–2 mg, i.p.), the abdominal cavity was opened, and the portal vein was catheterized. The liver was perfused with heparinized (10 U/ml) Hank's balanced salt solution (HBSS; GIBCO) without Ca^{2+} or Mg^{2+} for 5 min at a flow rate of 2.5 ml/min. The perfusate was then changed to HBSS containing 0.05% (w/v) collagenase type 1 (Sigma) and 0.05% (w/v) neutral protease (dispase, grade II; Boehringer Mannheim, Indianapolis, IN) for 10 min. The liver was then excised and teased apart in HBSS containing deoxyribonuclease (100 U/ml, type 1; Sigma). Leukocytes were isolated from hepatocytes by metrizamide density gradient centrifugation. Washed, and resuspended to the desired concentration. Nonadherent PEC and nonadherent liver leukocytes were obtained following a 1-hr adherence to plastic Petri dishes at 37°C.

Single-Cell Cytotoxicity Assay

To directly visualize cytolysis mediated by dividing cells, a single-cell agarose cytotoxicity assay combined with autoradiography was employed [8]. Briefly, pooled (from four to ten mice per group) effector cells (6×10^5) that had been pulse-labeled with ^{3}H-thymidine (New England Nuclear, Boston, MA) for 1 hr were used to form effector cell-target cell conjugates with K562 target cells (6×10^5). The conjugates were suspended in 0.5% agarose (equal volumes to type 1 and type VII; Sigma) medium and were spread on microscopic slides. The slides were incubated for 4 hr at 37°C in a moist environ-

ment to allow for cell-mediated cytotoxicity. Dead target cells were visualized by staining with 0.1% trypan blue (GIBCO) in PBS for 5 min. The slides were then washed, fixed in 0.2% formaldehyde, and dried at room temperature. Autoradiography using Kodak nuclear track emulsion type NTB2 (Eastman Kodak Company, Rochester, NY) was used to determine which effector cells had incorporated ^{3}H-thymidine during the pulse. Conjugates (600–700) that contained a single effector cell and a single target cell were scored per assay. The total percent cytotoxicity is equal to the fraction of single-cell conjugates that contained dead target cells × 100. The percent of killing mediated by blast cells is equal to (the number of single-cell conjugates containing dead target cells bound to blast cells ÷ the total number of single-cell conjugates containing dead target cells) × 100.

Cytotoxicity Assays

Target cells (10^6) were labeled with 100 μCi sodium chromate (^{51}Cr; Amersham Corp., Arlington Heights, IL) for 1 hr at 37°C, washed, and mixed with effector cells in quadruplicate in round-bottomed microtiter wells (Costar, Cambridge, MA) at 10^4 target cells per well in a total volume of 0.2 ml. For spontaneous release determinations, medium was added without effector cells. Nonidet P-40 (1%) was added for maximum release determinations. The plates were incubated for 4–16 hr at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. At the end of incubation, plates were centrifuged at 500 g for 5 min, and 0.1 ml of the supernatant was removed and counted in a Beckman gamma counter (Model 5500; Beckman Instruments, Palo Alto, CA). For the data presented, the coefficient of variation among quadruplicate wells was less than 5%. Data are expressed as:

% specific ^{51}Cr release:

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

In some experiments examining CTL activity, lysis is expressed as: % virus-specific lysis = % specific ^{51}Cr release for the infected target minus % specific ^{51}Cr release for the uninfected target.

To compare the total lytic capabilities of leukocyte populations isolated from different organs, we calculated total lytic units = (fraction of target cells killed × number of leukocytes per organ) ÷ E:T ratio. An E:T ratio on the linear portion of the cytotoxicity curve was used for these calculations.

Surgical Procedures

Mice anesthetized by injection of sodium pentobarbital (1–2 mg, i.p.) were shaved and swabbed with ethanol and iodine. A lateral incision was made through the abdominal wall, and the spleen was exteriorized on its pedicle, wrapped in sterile gauze moistened with saline, and immobilized. Following irradiation (see below), the spleen was replaced in the abdominal cavity, and the incision was closed with stainless steel surgical staples. For splenectomy, mice were similarly treated except that following exteriorization of the spleen, a silk suture ligature was tied around the pedicle, and the spleen was excised. Sham-operated mice underwent the same surgical treatment but without the application of the ligature or removal of the spleen.

Irradiation of Mice

Gamma irradiation was administered to mice in a Gammacell 40 (Atomic Energy of Canada Limited, Ottawa, Canada) equipped with ^{137}Cs sources at a dose rate of 125 R/min. Electron irradiation (20 MeV) was delivered by a Saturne 20 linear accelerator (Thomson-CGR, Columbia, MD) at 300 R/min. Dosimetry measurements conducted at the time of irradiation were made with ionization chambers (PTW Corporation, Freiburg, FRG) placed adjacent to or directly beneath the animal being irradiated. Shielding from electron irradiation was provided by inverted 5-mm-thick lead cups.

Fluorescence-Activated Cell Sorter (FACS)

Leukocytes were stained for FACS analysis and sorting using rat monoclonal antibody 53–6.7 [28] against Lyt-2 followed by F(ab')$_2$ mouse anti-rat immunoglobulin conjugated with fluorescein isothiocyanate (Jackson Immunoresearch Labs, Inc., Avondale, PA). Analysis and sorting were performed on a FACS 440 (Becton-Dickinson FACS Systems, Mountain View, CA) at a laser output of 300 mW at 488 nm with green fluorescence collected through a DF 530/30 filter. Cells were separated into fluorescence-negative and fluorescence-positive populations at a flow rate of 3–4 × 10^3 cells/sec.

Cell Morphology

Leukocytes (1–3 × 10^4) were centrifuged onto glass slides in a cytocentrifuge (Shandon Southern Instruments, Inc., Sewickley, PA) and stained with Wright's and Giemsa stains. Leukocytes (200–500) were counted under oil immersion microscopy for the enumeration of LGL percentages. The proportion of LGL exhibiting blast cell morphology (enlarged cell size, basophilic cytoplasm, and prominent nucleoli) [18] or of mitotic figures with cytoplasmic granules was determined by counting 100–200 LGL and classifying them into blast or nonblast LGL subsets.

Statistical Analyses

Data are presented as the mean ± standard error of the mean (SEM) for groups of two to five mice. Where no

TABLE 1. Effect of Irradiation on the NK/LGL Response in the Liver and Peritoneal Cavity to Viral Infection[a]

Treatment	Irradiation[b]	% YAC-1 lysis[c]	Total lytic units ($\times 10^4$)[d]	% Leukocytes with LGL morphology	Total LGL ($\times 10^4$)
Liver					
Experiment A					
Control	−	2.7 ± 0.1	1.5 ± 0.2	3.7 ± 0.9	14. ± 3.0
	+	4.0 ± 0.1	1.2 ± 0.1	4.7 ± 0.9	10. ± 2.0
LCMV-infected	−	39. ± 3.2	30. ± 4.9	9.0 ± 0.6	49. ± 1.0
	+	22. ± 3.5	3.2 ± 0.1	7.7 ± 0.9	8.0 ± 1.0
Peritoneal cavity					
Experiment A					
Control	−	< 1.0	< 0.1	< 0.3	< 1.0
	+	< 1.0	< 0.1	<0.3	< 0.4
LCMV-infected	−	27.	3.8	2.5	8.8
	+	2.9	0.1	2.0	1.7
Experiment B					
MHV-infected	−	26.	22.0	4.0	53.
	+	43.	0.9	6.5	2.2

[a]Liver leukocytes and PEC were obtained from C57BL/6J mice 3 days after irradiation and infection.
[b]1,000 R (experiment A) or 900 R (experiment B) gamma irradiation administered 1 hr prior to infection.
[c]4–5-hr assays; liver E:T = 7:1, PEC E:T = 25:1.
[d]Calculated as described in Materials and Methods.

TABLE 2. Effect of Shielding the Spleen Against Lethal Irradiation, or of Splenectomy, on the NK/LGL Response to MHV Infection[a]

	Treatment	%YAC-1 lysis[b]	Total leukocytes ($\times 10^5$)	% Leukocytes with LGL morphology	Total LGL ($\times 10^4$)
A. Irradiation					
Liver	Total body	19. ± 1.7	28. ± 3.3	20. ± 0.6	56. ± 8.0
	Spleen shielded	26. ± 5.8	35. ± 5.0	17. ± 1.3	60. ± 6.0
Peritoneal	Total body	29.	2.2	12.	2.6
cavity	Spleen shielded	18.	2.8	4.8	1.3
B. Splenectomy					
Liver	Sham-operated	27. ± 4.1	110. ± 6.9	7.3 ± 0.6	76. ± 9.0
	Splenectomized	31. ± 5.5	130. ± 9.0	7.0 ± 1.1	91. ± 11.
Peritoneal	Sham-operated	44. ± 8.0	350. ± 80.	13. ± 0.6[c]	150. ± 40.[d]
cavity	Splenectomized	33. ± 6.0	340. ± 90.	11. ± 1.0[c]	120. ± 20.[d]

[a]C57BL/6J mice received 900 R electron irradiation with or without shielding of their spleens (experiment A), or were sham-operated or splenectomized (experiment B), as described in Materials and Methods. Mice were then immediately infected with MHV, and liver leukocytes and PEC were obtained 3 days p.i.
[b]YAC-1 assays were 5 hr at E:T = 3:1 (experiment A), or 4 hr at liver E:T = 17:1 and PEC E:T = 33:1 (experiment B).
[c]Nonadherent PEC were used for cytocentrifuge preparations.
[d]Calculated on percentage (33%) of total leukocytes that were nonadherent.

SEM is shown, data were obtained from pooled leukocyte populations of two to five mice. Statistical significance between groups was determined by the Fisher exact test or Student's t test.

RESULTS

NK/LGL Response in Lethally Irradiated Mice

We have previously shown that viral infection results in 5–50-fold increases in NK/LGL numbers in the peritoneal cavity [36] and the liver [33]. To determine whether this accumulation of NK/LGL at sites of infection could occur in the absence of cell division, mice were lethally irradiated to prevent cellular proliferation and then infected with virus. The cellular immune response was monitored 3 days later. In uninfected control mice, 900–1,000 R gamma irradiation reduced by > 99% the viable leukocyte recoveries from bone marrow and spleen and reduced by > 95% the incorporation of ^{3}H-thymidine by bone marrow cells stimulated by L-

929 cell-conditioned medium (data not shown). Irradiation of uninfected control mice did not markedly alter NK cell activity, total lytic units, or LGL number in the liver (Table 1); the peritoneal cavities of uninfected mice contained virtually no NK cell activity or LGL, regardless of whether or not they had been irradiated. In contrast, preirradiation of mice reduced by 80–95% the total lytic capacities and LGL numbers in the liver and PEC leukocyte populations induced by LCMV (Table 1, experiment A). This was due to a marked inhibition in the increase in total leukocyte numbers. This irradiation-sensitive effect was even more pronounced following MHV infection (Table 1, experiment B), which routinely induces much greater increases in LGL and total NK cell activity (lytic units) in the peritoneal cavity than does the LCMV infection [36]. These findings suggested that the resident NK/LGL in the control mice were not rapidly dividing, but that cell proliferation was necessary for the accumulation of normal numbers of LGL (and other leukocyte classes) in response to viral infection.

Role of the Spleen in NK/LGL Response to MHV Infection

Since the spleen is a site of mature NK cell blastogenesis during viral infection [8], we hypothesized that it might provide a significant number of the LGL accumulating in the liver and peritoneal cavity. We examined the ability of the spleen alone to provide NK/LGL for the early immune response. Mice were anesthetized, and their spleens were isolated outside the abdominal wall under lead shielding. Electron irradiation (900 R) was then administered to these mice and to similarly operated mice without lead-shielded spleens. Dosimetry measurements indicated that the shielded spleens received less than 5% of the total dose of irradiation, and the shielded spleens contained 7–20-fold greater numbers of viable leukocytes than did the irradiated spleens at day 3 postirradiation (data not shown). Table 2 (experiment A) indicates that, in those animals whose spleens were spared from the irradiation, NK/LGL responses in the liver and peritoneal cavity to MHV infection were not significantly greater than those of animals whose spleens were irradiated. Thus preserving the proliferative capacity of splenic NK/LGL did not alter the deleterious effects of lethal irradiation of other lymphoid compartments. However, other effects of the irradiation, such as altered lymphocyte trafficking patterns or the generation of factors inhibiting proliferation of the viable cells in the spleen, could have accounted for these results. Because of this uncertainty, we used a splenectomy model to examine the contribution of NK/LGL made by the spleen. Mice were splenectomized or sham-operated and then immediately infected with MHV. At day 3 p.i., the absence of a spleen did not appreciably alter the NK/LGL response in either the liver or the peritoneal cavity (Table 2, experiment B). Thus, by two different methods, we could not demonstrate a significant role of the spleen in providing NK/LGL to these nonlymphoid compartments.

Blast LGL in Nonlymphoid Organs During Viral Infection

Viral infection or exposure to IFN in vivo elicits the blastogenesis of spleen NK cells with a concomitant increase in cell size [6,8]. Microscopic examination of stained cytocentrifuge smears of PEC and liver leukocytes from poly I:C-treated or virus-infected mice revealed the presence of LGL with pronounced size enlargement and intense cytoplasmic basophilia, characteristics of a blast cell morphology [18]. Following size separation of poly I:C-stimulated liver leukocytes by centrifugal elutriation [5,6,8], the majority (81%) of the LGL exhibiting blast cell morphology were recovered in the large cell fractions (fractions 4–6) (dat not shown). The small cell fractions (fractions 1–3) contained 61% of all nonblast LGL recovered and had few LGL that were blast LGL. These data support the use of morphological criteria to classify LGL into blast and nonblast populations and further indicate that the interferon inducer poly I:C elicited LGL with blast size and morphology in the liver. The majority of LGL in the liver [33] and peritoneal cavity [36] at this time postinfection (day 3) have been well characterized as NK cells, suggesting that the blast LGL described here were NK/LGL.

The presence of blast LGL in the leukocytes isolated from liver and peritoneal cavity was examined in mice infected with different viruses. Viral infection resulted in significant increases in both the percentages and total number of blast LGL at day 3 p.i. in these nonlymphoid compartments, with cytopathic viruses (e.g., MHV and vaccinia virus) generally producing the greatest increases (Table 3). The blast (and nonblast) LGL at day 3 p.i. were significantly depleted ($P < 0.05$) by in vivo treatment with anti-asialo GM_1 serum, further substantiating their NK cell phenotype. At day 3 p.i., similar blast NK/LGL were also found in athymic nude mice as well as in beige mice, whose lymphocytes have abnormal granule morphology and deficient cytotoxic capacity [21,39].

Thymidine-Incorporating Killer Cells in the Liver and Peritoneal Cavity

The presence of morphologically defined blast LGL in leukocyte populations freshly isolated from the liver and peritoneal cavity during viral infection suggested that cytotoxic lymphocytes were proliferating at these sites. To confirm that the specific cells mediating the killing were indeed blast cells, nonadherent leukocytes from livers and peritoneal cavities of control mice and MHV-infected mice at day 3 p.i. were labeled in vitro with ^3H-

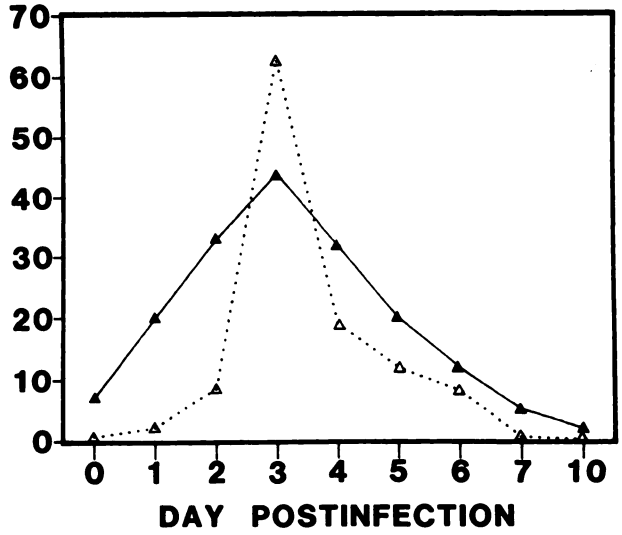

Fig. 1. Kinetics of LGL blastogenesis during MHV infection. Mice (n = 2–4 per group) were infected i.p. with MHV, and PEC were prepared at the indicated times postinfection. Percent of the LGL exhibiting blast morphology (▲—▲); number of blast LGL (× 10^4) per animal (△ - - -△).

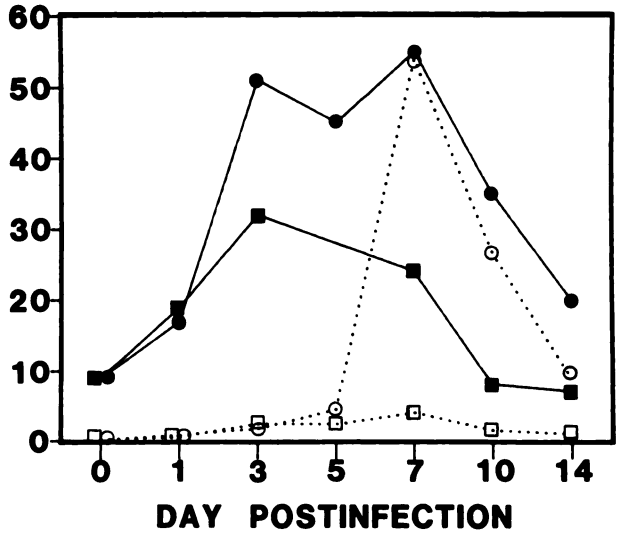

Fig. 2. Kinetics of LGL blastogenesis during LCMV infection. Mice (n = 2–4 per group) were infected i.p. with LCMV-ARM (■, □) or LCMV-WE (●, ○), and liver leukocytes were prepared at the indicated times postinfection. Percent of the LGL exhibiting blast morphology (—). Number of blast LGL (× 10^5) per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

thymidine and tested as effector cells in single-cell cytotoxicity assays with autoradiography. The data in Table 4 show that MHV infection induced significant increases in NK cell activity (YAC-1 lysis) and in the percentage of LGL with blast cell morpholgy. Leukocytes from infected mice that had bound a target were more lytically active on a per cell basis (% killing in conjugates), compared with leukocytes from control mice. Most importantly, a significantly ($P < 0.001$; Fisher exact test) greater percentage of the lytic activity on NK cell-sensitive targets was mediated by ^{3}H-thymidine-incorporating effector cells from infected mice than from uninfected mice. These data show that leukocytes freshly isolated from these nonlymphoid compartments at day 3 p.i. contain ^{3}H-thymidine-incorporating, functionally active, blast NK cells.

Kinetics of LGL Blastogenesis

The kinetics of LGL blastogenesis in the peritoneal cavity were examined during MHV infection. Both the percent of the LGL that were blasts and the total numbers of blast LGL in the peritoneal cavity peaked at day 3 p.i. and then declined rapidly (Fig. 1). By day 7 p.i. with MHV, LGL blastogenesis and total LGL number had returned to control values.

The kinetics of LGL blastogenesis in the liver following infection of C3H/St mice with the hepatotropic WE strain of LCMV (LCMV-WE) or the nonhepatotropic Armstrong strain of LCMV (LCMV-ARM) had a different pattern. Increases in blast LGL proportions were evident as early as day 1 p.i. with both viruses (Fig. 2), but, in contrast to MHV-induced blastogenesis, LCMV-induced blastogenesis remained elevated through day 7 p.i. before declining. This was a difference between LCMV and MHV, not of liver and peritoneal cavity, as the peritoneal LGL blast cell response to LCMV-ARM resembled that of the liver (data not shown). Compared with infection with LCMV-ARM, infection with LCMV-WE resulted in significantly ($P < 0.02$) higher percentages of blast LGL in liver leukocytes by day 3 p.i., and this difference persisted through day 14 p.i. The total number of blast LGL in livers infected with either LCMV-ARM or LCMV-WE peaked at day 7 p.i., but these levels were 13-fold higher following hepatotropic LCMV-WE infection (Fig. 2).

Cell Surface Phenotype of Blast LGL

We examined by FACS the blast LGL populations from the liver and peritoneal cavity of mice at days 3 and 7 p.i. with LCMV for expression of the CTL-specific marker Lyt-2. At day 3 p.i., the majority of the LGL (77%), blast LGL (81%), and NK cell activity was found in Lyt-2^- FACS-sorted populations from the liver (Table 5). By contrast, most LGL (58%) and blast LGL (82%) in the liver at day 7 p.i. were Lyt-2^+. NK cell activity at day 7 p.i. was mediated exclusively by Lyt-2^- cells. Lyt-2^+ cells, which were devoid of CTL activity at day 3 p.i., expressed high levels of virus-specific (CTL) lysis at day 7 p.i. (Table 5). Similar data were obtained for the PEC: 94% of the blast LGL at day 3 p.i. were Lyt-

TABLE 3. Blast LGL in the Liver and Peritoneal Cavity 3 Days After Viral Infection[a]

Mouse strain	Treatment	% LGL with blast morphology	Total blast LGL ($\times 10^4$)
Liver			
C57BL/6J	Control	5.5 ± 1.3	0.7 ± 0.2
	MHV	44. ± 0.3	53. ± 15.
	MHV + anti-AGM1	55. ± 8.7	8.5 ± 3.7
C57BL/6J	Control	8.5 ± 3.5	2.5 ± 0.8
	MCMV	70. ± 2.1	149. ± 21.
C57BL/6J	LCMV-ARM	31. ± 2.3	25. ± 2.2
	LCMV-ARM + anti-AGM1	31. ± 3.7	7.2 ± 5.9
C57BL/6J			
bg/bg	Control	4.3 ± 1.0	3.6 ± 1.0
bg/bg	MHV	42. ± 3.1	188. ± 27.
BALB/c			
nu/nu	Control	5.0 ± 2.8	1.9 ± 0.6
nu/nu	LCMV-ARM	50. ± 5.0	54. ± 17.
nu/nu	Poly I:C	59. ± 2.0	52. ± 11.
Peritoneal cavity[b]			
C57BL/6J	Control	<5.0	<0.05
	MHV	43.	23.
	MHV + anti-AGM1	33.	0.8
C57BL/6J	MCMV	26.	2.8
C57BL/6J	Vaccinia virus	25.	24.
C57BL/6J	LCMV-ARM	28.	3.9
C3H/St	Pichinde virus	33.	3.9

[a]Cytocentrifuge preparations of leukocytes from the livers and peritoneal cavities of mice (n = 3–5 per group) at day 3 postinfection were examined for the proportion of LGL with blast cell morphology, as described in Materials and Methods.
[b]The data presented were obtained from PEC populations pooled from three to five mice.

2^-, whereas the vast majority (91%) of the blast LGL at day 7 p.i. were Lyt-2^+. Even though Lyt-2^- LGL, presumed to be NK cells, were still present at day 7 p.i., few of them were dividing.

We also examined the sensitivity of blast LGL at day 7 p.i. with LCMV to in vivo anti-asialo GM_1 treatment. In contrast to the demonstrated sensitivity of day 3 p.i. blast LGL and NK cell activity to anti-asialo GM_1 (Table 3), two treatments with antiserum at days 0 and 4 p.i. reduced neither the total number of blast LGL nor the level of CTL-mediated virus-specific lysis, although the low level of NK cell activity normally present at day 7 p.i. was reduced (data not shown). These data indicate that LGL blastogenesis shifted from NK cell-rich, Lyt-2^-, anti-asialo GM_1-sensitive leukocytes at day 3 p.i. to CTL-rich, Lyt-2^+, anti-asialo GM_1-resistant leukocytes at day 7 p.i.

DISCUSSION

This report documents that the previously observed [33,36] nonlymphoid organ-associated increases in NK/LGL and CTL/LGL at early and late stages of viral infections, respectively, are associated with high levels of blastogenesis within these populations. At one time NK cells were thought to be end-stage cells because of their relative resistance to irradiation [19,23]. This resistance, however, was a property of endogenous, unstimulated NK cells, few of which are blasts [6]. IFN stimulation converts the NK cell population to a rapidly dividing, blast cell-enriched population [6,8]. Studies presented here reaffirm that irradiation has minimal short-term effects on endogenous NK/LGL and also show that irradiation greatly reduces the NK/LGL infiltrate in the organs of virus-infected mice (Table 1). This suggests that cell division is required for the accumulation of these cells.

The presence of large numbers of blast NK/LGL and blast CTL/LGL in virus-infected organs raises the question as to their origin. We hypothesized that the spleen would be a prominent source of NK/LGL for peripheral target organs, since mature NK cells proliferate in the spleen during viral infection [8]. However, neither splenectomy nor shielding the spleen during irradiation yielded any evidence implicating the spleen as the major contributor of the NK/LGL response in the liver and

TABLE 4. Thymidine-Incorporating NK Cells in the Liver and Peritoneal Cavity During Viral Infection

	% YAC-1 lysis[a]	% Leukocytes with LGL morphology	% LGL with Blast morphology	^{3}H-thymidine incorporation (CPM)[b]	% Cytotoxicity in conjugates[c]	% Killing mediated by ^{3}H-labeled effector cells
Liver						
Control	16.	12.	20.	2,800	13.	14.
MHV	50.	38.	56.	5,700	30.	25.
Peritoneal cavity						
Control	2.5	1.	<1.0	1,100	10.	2.8
MHV	28.	33.	14.	7,200	30.	33.

[a]Four-hr assay; liver E:T = 3:1, PEC E:T = 3:1.
[b]CPM/10^5 nonadherent leukocytes following 1-hr pulse with ^{3}H-thymidine.
[c]From single-cell assays as described in Materials and Methods.

peritoneal cavity (Table 2). NK cell precursors are at high levels in the bone marrow, and this compartment may contribute some of the NK cells that accumulate in organs. The recent observation (R. Wiltrout, personal communication) that depletion of bone marrow by ^{89}Sr reduces the accumulation of NK/LGL in the liver following treatment of mice with the biological response modifier MVE-2 lends support to this possibility. Cell division could also be taking place in these peripheral organs, and/or cells with blast morphology could be preferentially attracted to or trapped within these compartments. Data presented in this report indicate that a significant proportion of NK cells freshly isolated from the virus-infected organs are ^{3}H-thymidine-incorporating effector cells (Table 4). This shows that NK cells are, in fact, capable of at least one round of proliferation in nonlymphoid organs but does not rule out the possibility that many of the blast LGL have recently migrated into the virus-infected organs from other compartments.

The peritoneal cavities of uninfected mice have very few NK/LGL (Tables 1,3, and 4), and adoptive transfer of PEC from normal mice into the peritoneal cavities of virus-infected irradiated recipients failed to reconstitute the PEC NK/LGL response (data not shown). Thus it appears that NK/LGL or their precursors must first migrate into the peritoneal cavity, where their continued proliferation can contribute to the local immune response. Consistent with this proposal, we have recently shown that virus-induced peritoneal NK/LGL and CTL/LGL have the ability to chemotactically respond in vitro to factors produced at sites of viral infection [37]. Furthermore, the majority of these LGL migrating in vitro exhibit the blast LGL morphology [37]. The normal liver, however, contains a resident population of functional NK/LGL (Tables 1, 3 and 4) [33,48], and a proportion of these cells is probably induced to proliferate in situ in response to local or systemic stimuli.

Other experiments indicate that, following adoptive transfer, antigen-sensitized blast lymphocytes migrate into sites of inflammation more readily than do small lymphocytes [3,31,34]. This preferential accumulation is apparently not antigen-specific [3,31,34] and may be at least partially explained by the blast cells' greatly increased motility [20]. However, a preferential accumulation of the blast lymphocytes that *are* generated during a developing immune response may provide a means for the selective concentration of both nonspecific (NK/LGL) and antigen-specific (CTL/LGL) blast effector cells at the sites of antigenic stimulation.

CTL precursors proliferate in vivo in response to viral [1,5,7,8] and allogeneic [12] antigens, and they can differentiate into cytolytic cells at a site of localized antigenic stimulation [2]. At day 3 p.i. with LCMV, the liver and peritoneal cavity have few Lyt-2^+ cells (Table 5). By day 7 p.i., the proportion of Lyt-2^+ cells increases dramatically in these compartments, and high percentages of the Lyt-2^+ LGL display blast cell morphology. The presence of Lyt-2^+ blast LGL strongly suggests that CTL are also capable of at least limited proliferation in these organs. The extensive replication of LCMV-WE in the liver [46] probably serves as a potent stimulus for the blastogenesis of LGL, accounting in part for the greater numbers of total CTL/LGL [33] and blast CTL/LGL (Fig. 2) at day 7 p.i. than are seen following nonhepatotropic LCMV-ARM infection. With the demonstration of the critical importance of CTL in mediating clearance of infection in the LCMV model system [32,49], the high levels of localized blastogenesis and potential proliferation of these cytotoxic effector cells within the liver itself, especially during an hepatotropic infection, are quite significant. In contrast, MHV infection fails to elicit a demonstrable virus-specific CTL response [43] and does not induce the accumulation of large numbers of LGL in infected organs at day 7 p.i. [36]. These observations are

TABLE 5. Lyt-2 Phenotype of Blast LGL at Days 3 and 7 Postinfection With LCMV-ARM

Cell population[a]	% Leukocytes with LGL morphology[b]	% LGL with blast morphology[c]	% Virus-specific lysis[d]	% VAC-1 lysis[e]
Liver				
Day 3				
Lyt-2$^+$ (11)	34. (23)	34. (19)	<1.0	11.
Lyt-2$^-$ (89)	13. (77)	48. (81)	<1.0	20.
Day 7				
Lyt-2$^+$ (27)	42. (58)	44. (86)	60.	6.2
Lyt-2$^-$ (63)	13. (42)	10. (14)	16.	32.
Peritoneal cavity				
Day 3				
Lyt-2$^+$ (4.4)	3.0 (2.7)	79. (6.3)	ND[f]	ND
Lyt-2$^-$ (96)	5.0 (97)	32. (94)	<1.0	15.
Day 7				
Lyt-2$^+$ (50)	33. (75)	12. (91)	13.	0.3
Lyt-2$^-$ (50)	11. (25)	3.5 (8.8)	4.3	9.7

[a]Leukocytes were obtained from the livers and peritoneal cavities of C3H/St mice at days 3 and 7 p.i. with LCMV-ARM. Liver leukocytes and nonadherent PEC were stained with anti-Lyt-2 antibody and sorted by FACS as described in Materials and Methods. Values in parentheses show the percent distribution of leukocytes within the indicated Lyt-2 phenotypes.

[b]Percent of the FACS-sorted cells with LGL morphology. Values in parentheses show the percent of all LGL exhibiting the indicated Lyt-2 phenotype.

[c]Percent of the FACS-sorted LGL with blast cell/mitotic figure morphology. Values in parentheses show the percent of all blast LGL exhibiting the indicated Lyt-2 phenotype.

[d]Percent virus-specific lysis is the difference in lysis between LCMV-infected and uninfected L-929 cells in 14-hr assays at E:T = 10:1.

[e]YAC-1 assays were run for 4 hr at E:T = 10:1.

[f]Not determined (insufficient number of cells recovered).

supported by the absence of significant LGL blastogenesis late in MHV infection (Fig. 1), when CTL would otherwise be expected to be undergoing proliferation.

In summary, we have demonstrated that dividing NK cells are present in nonlymphoid organs of virus-infected mice. Increases in NK/LGL number are sensitive to irradiation and are strongly associated with increased proportions of LGL with blast cell morphology and with killing mediated by ^{3}H-thymidine-incorporating NK cells. In viral infections (such as LCMV) that induce a potent CTL response, CTL/LGL blastogenesis also occurs in nonlymphoid organs, peaking later in infection. This blastogenesis and proliferation of cytotoxic effector cells at localized sites of viral replication may provide a mechanism for in situ amplification of these crucial components of the host's antiviral defenses.

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REFERENCES

1. Allan, J.E., and Doherty, P.C. Consequences of cyclophosphamide treatment in murine lymphocytic choriomeningitis: Evidence for cytotoxic T cell replication *in vivo*. Scand. J. Immunol. 22,367, 1985.
2. Ascher, N.L., Chen, S., Hoffman, R., and Simmons, R.L. Maturation of cytotoxic effector cells at the site of allograft rejection. Transplant. Proc. 13,1105, 1981.
3. Asherson, G.L., Allwood, G.G., and Mayhew, B. Contact sensitivity in the mouse. XI. Movement of T blasts in the draining lymph nodes to sites of inflammation. Immunology 28,485, 1973.
4. Bainton, D.F., Ullyot, J.L., and Farquhar, M.G. The development of neutrophilic polymorphonuclear leukocytes in human bone marrow. Origin and content of azurophil and specific granules. J. Exp. Med. 134,907, 1971.
5. Biron, C.A., Natuk, R.J., and Welsh, R.M. Generation of large granular T lymphocytes *in vivo* during viral infection. J. Immunol. 136,2280, 1986.
6. Biron, C.A., Sonnenfeld, G., and Welsh, R.M. Interferon induces natural killer cell blastogenesis *in vivo*. J. Leuk. Biol. 35,31, 1984.
7. Biron, C.A., Turgiss, L.R., and Welsh, R.M. Increase in NK cell number and turnover rate during acute viral infection. J. Immunol. 131,1539, 1983.
8. Biron, C.A., and Welsh, R.M. Blastogenesis of natural killer cells during viral infection *in vivo*. J. Immunol. 129,2788, 1982.
9. Buchmeier, M.J., Lewicki, H.A., Tomori, O., and Oldstone, M.B.A. Monoclonal antibodies to lymphocytic choriomeningitis

and Pichinde viruses: Generation, characterization, and cross-reactivity with other arenaviruses. Virology 113,73, 1981.
10. Bukowski, J.F., Warner, J.F., Dennert, G., and Welsh, R.M. Adoptive transfer studies demonstrating the antiviral effect of natural killer cells *in vivo*. J. Exp. Med. 161,40, 1985.
11. Bukowski, J.F., Woda, B.A., Habu, S., Okumura, K., and Welsh, R.M. Natural killer cell depletion enhances virus synthesis and virus-induced hepatitis *in vivo*. J. Immunol. 131,1531, 1983.
12. Denizot, F., Wilson, A., Battye, F., Berke, G., and Shortman, K. Clonal expansion of T cells: A cytotoxic T-cell response *in vivo* that involves precursor cell proliferation. Proc. Natl. Acad. Sci. 83,6089, 1986.
13. Edwards, J.L. and Klein, R.E. Cell renewal in adult mouse tissues. Am. J. Pathol. 38,437, 1961.
14. Fenyo, E.M., Klein, E., Klein, G., and Swiech, K. Selection of an immunoresistant Moloney lymphoma subline with decreased concentration of tumor-specific surface antigens. J. Natl. Cancer Inst. 40,69, 1968.
15. Habu, S., Fukui, H., Shimamura, K., Kasai, M., Nagai, Y., Okumura, K., and Tamaoki, N. *In vivo* effects of anti-asialo GM1. I. Reduction of NK activity and enhancement of transplanted tumor growth in nude mice. J. Immunol. 127,34, 1981.
16. Haller, O., Kiessling, R., Orn, A., and Wigzell, H. Generation of natural killer cells: An autonomous function of the bone marrow. J. Exp. Med. 145,1411, 1977.
17. Haller, O., and Wigzell, H. Suppression of natural killer cell activity with radioactive strontium: Effector cells are marrow dependent. J. Immunol. 118,1503, 1977.
18. Hayry, P., and von Willebrand, E. Practical guidelines for fine needle aspiration biopsy of human renal allografts. Ann. Clin. Res. 13,288, 1981.
19. Hochman, P.S., Cudkowicz, G., and Dausset, J. Decline of natural killer cell activity in sublethally irradiated mice. J. Natl. Cancer Inst. 61,265, 1978.
20. Hoffman, R.A., Ascher, N.L., and Simmons, R.L. Migratory behavior of lymphocytes during allograft rejection. Transplant. Proc. 13,1102, 1981.
21. Itoh, K., Suzuki, R., Umezu, Y., Kanaumi, K., and Kumagai, K. Studies of murine large granular lymphocytes. II. Tissue, strain, and age distributions of LGL and LAL. J. Immunol. 129,395, 1982.
22. Kelly, L.S., Brown, B.A., and Dobson, E.L. Cell division and phagocytic activity in liver reticulo-endothelial cells. Proc. Soc. Exp. Biol. Med. 110,555, 1962.
23. Kiessling, R., Hochman, P.S., Haller, O., Shearer, G.M. Wigzell, H., and Cudkowicz, G. Evidence for a similar or common mechanism for natural killer cell activity and resistance to hemopoietic grafts. Eur. J. Immunol. 7,655, 1977.
24. Kiessling, R., Klein, E., and Wigzell, H. "Natural " killer cells in the mouse. I. Cytotoxic cells with specificity for mouse Moloney leukemia cells. Specificity and distribution according to genotype. Eur. J. Immunol. 5,112, 1975.
25. Kincade, P.W. Formation of B lymphocytes in fetal and adult life. Adv. Immunol. 31,177, 1981.
26. Kumagai, K., Itoh, K., Suzuki, R., Hinuma, S., and Saitoh, F. Studies of murine large granular lymphocytes. I. Identification as effector cells in NK and K cytotoxicities. J. Immunol. 129, 388, 1982.
27. Kumar, V., Ben-Ezra, J., Bennett, M., and Sonnenfeld, G. Natural killer cells in mice treated with ^{89}strontium: Normal target-binding cell numbers but inability to kill even after interferon administration. J. Immunol. 123,1832, 1979.
28. Ledbetter, J.A., and Herzenberg, L.A. Xenogeneic monoclonal antibodies to mouse lymphoid differentiation antigens. Immunol. Rev. 47,63, 1979.
29. Lozzio, C.B., and Lozzio, B.B. Human chronic myelogenous leukemia cell-line with positive Philadelphia chromosome. Blood 45,321, 1975.
30. Maloney, M.A., Weber, C.L., and Patt, H.M. Myelocyte-metamyelocyte transition in the bone marrow of the dog. Nature 197,150, 1963.
31. McGregor, D.D., and Logie, P.S. The mediator of cellular immunity. VII. Localization of sensitized lymphocytes in inflammatory exudates. J. Exp. Med. 139,1415, 1974.
32. McIntyre, K.W., Bukowski, J.F., and Welsh, R.M. Exquisite specificity of adoptive immunization in arenavirus-infected mice. Antiviral Res. 5,299, 1985.
33. McIntyre, K.W., and Welsh, R.M. Accumulation of natural killer and cytotoxic T large granular lymphocytes in the liver during virus infection. J. Exp. Med. 164,1667, 1986.
34. Moore, A.R., and Hall, J.G. Nonspecific entry of thoracic duct immunoblasts into intradermal foci of antigens. Cell. Immunol. 8,112, 1973.
35. Natuk, R.J., and Holowczak, J.A. Vaccinia virus proteins on the plasma membrane of infected cells. III. Infection of peritoneal macrophages. Virology 147,354, 1985.
36. Natuk, R.J., and Welsh, R.M. Accumulation and chemotaxis of natural killer/large granular lymphocytes at sites of virus replication. J. Immunol. 138,877, 1987.
37. Natuk, R.J. and Welsh, R.M. Chemotactic effect of human recombinant interleukin-2 on mouse activated large granular lymphocytes. J. Immunol. 139,2737, 1987.
38. Nedrud, J.G., Collier, A.M., and Pagano, J.S. Cellular basis for susceptibility to mouse cytomegalovirus: Evidence from tracheal organ culture. J. Gen. Virol. 45,737, 1979.
39. Roder, J., and Duwe, A. The beige mutation in the mouse selectively impairs natural killer cell function. Nature 278,451, 1979.
40. Sturman, L.S., and Holmes, K.V. Molecular biology of coronaviruses. II. Structure and Organization of virions. Adv. Virus Res. 39,112, 1983.
41. Stutman, O. Two main features of T-cell development: Thymus traffic and post-thymic maturation. Contemp. Top. Immunobiol. 7,1, 1977.
42. Timonen, T., Ranki, A., Saksela, E., and Hayry, P. Human natural killer cell-mediated cytotoxicity against fetal fibroblasts. III. Morphological and functional characterization of the effector cells. Cell. Immunol. 48,121, 1979.
43. Wege, H., Siddell, St., and ter Meulen, V. The biology and pathogenesis of coronaviruses. Curr. Top. Microbiol. Immunol. 99,165, 1982.
44. Welsh, R.M. Cytotoxic cells induced during lymphocytic choriomeningitis virus infection of mice. I. Characterization of natural killer cell induction. J. Exp. Med. 148,163, 1978.
45. Welsh, R.M., Biron, C.A., and Bukowski, J.F. Interplay between natural killer cells and virus infections. In: Microbiology 1984 (Leive, L., and Schlessinger, D., Eds.) Washington, D.C.: American Society for Microbiology, p. 320, 1984.
46. Welsh, R.M., Biron, C.A., Bukowski, J.F., McIntyre, K.W., and Yang, H. Role of natural killer cells in virus infections of mice. Surv. Synth. Pathol. Res. 3,409, 1984.
47. Welsh, R.M., Lampert, P.W., Burner, P.A., and Oldstone, M.B.A. Antibody-complement interactions with purified lymphocytic chorionmeningitis virus. Virology 73,59, 1976.
48. Wiltrout R.H., Mathieson, B.J., Talmadge, J.E. Reynolds, C.W., Zhang, S.R. Herberman, R.B., and Ortaldo, J.R. Augmentation of organ-associated natural killer activity by biological response modifiers. Isolation and characterization of large granular lymphocytes from the liver. J. Exp. Med. 160,1431, 1984.
49. Zinkernagel, R.M., and Welsh, R.M. H-2 compatibility requirement for virus-specific T cell-mediated effector functions *in vivo*. I. Specificity of T cells conferring antiviral protection against lymphocytic choriomeningitis virus is associated with H-2K and H-2D. J. Immunol. 117,1495, 1976.