ACCUMULATION OF NATURAL KILLER AND CYTOTOXIC T LARGE GRANULAR LYMPHOCYTES IN THE LIVER DURING VIRUS INFECTION

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Large granular lymphocytes (LGL)[1] are cytotoxic leukocytes with reniform nuclei and large cytoplasms containing azurophilic granules (1). These granules contain cytotoxic moieties that are capable of mediating target cell death in vitro (2, 3). Natural killer (NK) cells are LGL that have been implicated in the early resistance to certain virus infections in murine systems (4). Cytotoxic T lymphocytes (CTL) are important later in infection for the clearance of virus and recovery of the host (5). Recent work has demonstrated that the CTL generated in vivo during virus infection acquire the LGL morphology (6).

The activities of NK cells and CTL have been extensively studied in the spleens of mice during infection with several viruses, including lymphocytic choriomeningitis virus (LCMV) (7). LCMV-induced interferon (IFN) leads to significant activation (8), blastogenesis, and proliferation of NK cells, all of which peak in the spleen about 3 d postinfection (p.i.) (9–11). The activity and blastogenesis of spleen CTL peak later during infection (day 7 p.i.) (9, 10), and this killing is mediated by effector cells that are virus-specific and restricted in their killing function by class I antigens of the major histocompatibility complex (MHC) (12).

The presence of NK cells and CTL in other compartments of the virus-infected host has been less well studied. In fact, there has not been a demonstration by morphology of an accumulation of either NK/LGL or CTL/LGL in diseased organs. CTL and NK cell activities have been found in the cerebrospinal fluid during vaccinia virus–induced meningitis (13), and CTL are present in meningeal exudates during LCMV-induced meningitis (14). CTL have also been isolated from the lung during infection with influenza (15) and respiratory syncytial (16) viruses, but to our knowledge, virus-specific CTL have not been demonstrated in the virus-infected liver.

The liver is a major organ for the replication of many viruses and the sole organ for the replication of some. Virus-induced hepatitis is frequently a severe life-threatening disease affecting millions of people worldwide, but little is known about the cytotoxic inflammatory cell population within the diseased liver. Wiltrout et al. (17) have shown the presence of NK/LGL in the livers of mice,

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[1] *Abbreviations used in this paper:* asialo GM_1, ganglio-tetraosyceramide; GpC, guinea pig complement; LCMV, lymphocytic choriomeningitis virus; LCMV-ARM, Armstrong strain of LCMV; LCMV-WE, WE strain of LCMV; LGL, large granular lymphocyte; MCMV, murine cytomegalovirus; MHV, mouse hepatitis virus; p.i., postinfection; PV, Pichinde virus; RbC, rabbit complement.

and the NK cell activity and LGL number were significantly augmented after administration of certain biological response modifiers. In this report we document for the first time that high numbers of LGL accumulate in the liver during viral infection. An initial wave of NK/LGL is followed by a substantially greater influx of CTL/LGL. We compare hepatotropic and nonhepatotropic strains of LCMV (a noncytopathic virus) for their effect on NK cell and CTL accumulation and activity in the liver. The effect on NK/LGL accumulation stimulated by two highly cytopathic hepatotropic viruses, mouse hepatitis virus (MHV) and murine cytomegalovirus (MCMV), is contrasted to that caused by noncytopathic LCMV.

Materials and Methods

Mice. Mice were used between the ages of 6 and 12 wk. C3H/St male mice were purchased from West Seneca Laboratories (West Seneca, NY). C57BL/6J, C57BL/6J beige (*bg/bg*), and BALB/cJ mice were purchased from The Jackson Laboratories (Bar Harbor, ME). Athymic nude (*nu/nu*) and euthymic (*nu/+* mice (BALB/c background) were provided by Dr. C. Biron of our institution.

Viruses. The hepatotropic WE strain (18) of LCMV (LCMV-WE), the nonhepatotropic Armstrong strain of LCMV (LCMV-ARM), and Pichinde virus (PV), an arenavirus related to LCMV, were grown in L-929 cells (8). MHV, A59 strain (19), was a liver-grown stock propagated in vivo, and MCMV, Smith strain (4), 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 was injected intravenously (i.v.) (3×10^5 PFU), and MCMV (10^4 PFU) was injected i.p.

Cells. YAC-1 cells were derived from a Moloney leukemia virus–induced lymphoma in A/Sn mice (20, 21). These cells were maintained in RPMI 1640 medium (Gibco, Grand Island, NY) supplemented with antibiotics, glutamine, and 10% heat-inactivated FCS (M.A. Bioproducts, Walkerville, MD). L-929 cells, a continuous liver cell line from C3H mice, and the MC57G cell line from C57BL/6 mice, were maintained in minimum 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 h before use.

Reagents. Poly(I-C); Sigma Chemical Co., St. Louis, MO) was injected i.p. (100 μg) 2 d before use of mice.

Antisera. Rabbit anti–ganglio-tetraosylceramide (anti–asialo GM_1) (Wako Chemicals, Dallas, TX) was used to deplete NK cell activity selectively in vivo and in vitro (22, 23). For in vivo use, anti–asialo GM_1 was diluted 1:10 with RPMI medium, and 0.2 ml was injected i.v. Anti–asialo GM_1 was used in vitro at a dilution of 1:50. Monoclonal anti-Thy-1.2, clone HO-13-4 (24) (used diluted 1:1,500), and J11d antibody (25) (used undiluted) were generously provided by Dr. R. Woodland (University of Massachusetts Medical Center). Rat monoclonal antibody to Lyt-2, clone 53-6.7 (24), was used undiluted. Fresh guinea pig serum (GpC) and rabbit serum (RbC) (Pel-Freeze Biologicals, Brown Deer, WI) were used as sources of complement (C). Effector cells (see below) were treated with antibody reagent for 30 min at 4°C and then washed. In antibody-plus-C depletion studies, cells treated with anti–Lyt-2 were further treated (to improve C fixation) with a 1:50 dilution of monoclonal mouse anti–rat immunoglobulin, MAR 18.5 (26), for an additional 30 min at 4°C and then washed. All cells were resuspended in C for 30 min at 37°C, washed, resuspended to equal volume, and used in cytotoxicity assays (see below).

Preparation of Effector Cells. Spleen cells were obtained by gently dissociating aseptically removed spleens between two glass microscope slides. Erythrocytes were lysed by treatment with 0.84% ammonium chloride. The leukocytes were washed, counted, and resuspended in serum-containing medium at the desired concentration.

Effector cells were prepared from livers after in situ enzyme perfusion (27) with modifications. Mice were anesthetized with sodium pentobarbital (1 mg, i.p.), the abdominal cavity was exposed by a ventral midline incision, and the intestines were lifted to expose the portal vein. A silk suture ligature was loosely placed around the portal vein 0.5 cm from the liver. The portal vein was then catheterized with polyethylene tubing

(0.58 mm inner diam, 0.965 mm outer diam; Becton, Dickinson & Co., Parsippany, NJ), the ligature was tightened, and the abdominal aorta was transected. Perfusion (2.5 ml/min, 5 min) was begun with heparinized (10 U/ml), Ca^{2+}- and Mg^{2+}-free HBSS (Gibco) at 37°C. This initial perfusion served to flush peripheral blood from the organ. Only livers that were well blanched were used for further study. The perfusate was changed to prewarmed HBSS containing 0.05% (wt/vol) collagenase (type 1; Sigma Chemical Co.) and 0.05% (wt/vol) neutral protease (dispase, grade II; Boehringer Mannheim, Indianapolis, IN), and the perfusion was continued for an additional 10 min. The liver was then excised and teased apart with forceps in HBSS containing deoxyribonuclease (100 U/ml, type 1; Sigma Chemical Co.) on ice for 15 min. The liver cell suspension was passed through nylon mesh to remove clumps and pelleted at 1,000 *g*. The pellet was resuspended in minimal volume (0.5–0.75 ml) and mixed with isotonic 30% metrizamide (Sigma Chemical Co.) in Gey's solution (Gibco) at a ratio of seven parts metrizamide solution to five parts liver cell suspension (17). This mixture was transferred to a centrifuge tube, overlaid with 2 ml of PBS, and centrifuged for 20 min at 1,500 *g* at 10° C. The nonparenchymal cell layer was harvested from the interface, washed, counted, and resuspended to the desired concentration. This procedure routinely yielded at least $2–5 \times 10^6$ nonparenchymal cells per liver from control mice.

Fluorescence-activated Cell Sorting (FACS). Liver leukocytes were stained for FACS analysis and were sorted using rat monoclonal antibody 53-6.7 against Lyt-2 followed by $F(ab')_2$ mouse anti–rat immunoglobulin conjugated with fluorescein isothiocyanate (Jackson Immunoresearch Laboratories, Inc., Avondale, PA). Analysis and sorting were performed on a FACS 440 (Becton-Dickinson FACS Systems, Mountain View, CA) at a laser output of 300 mW at 488 nm with green fluorescence collected through a DF 530/30 filter. Cells were separated into fluorescence-negative and fluorescence-positive populations at a flow rate of $2–2.5 \times 10^3$ cells/s.

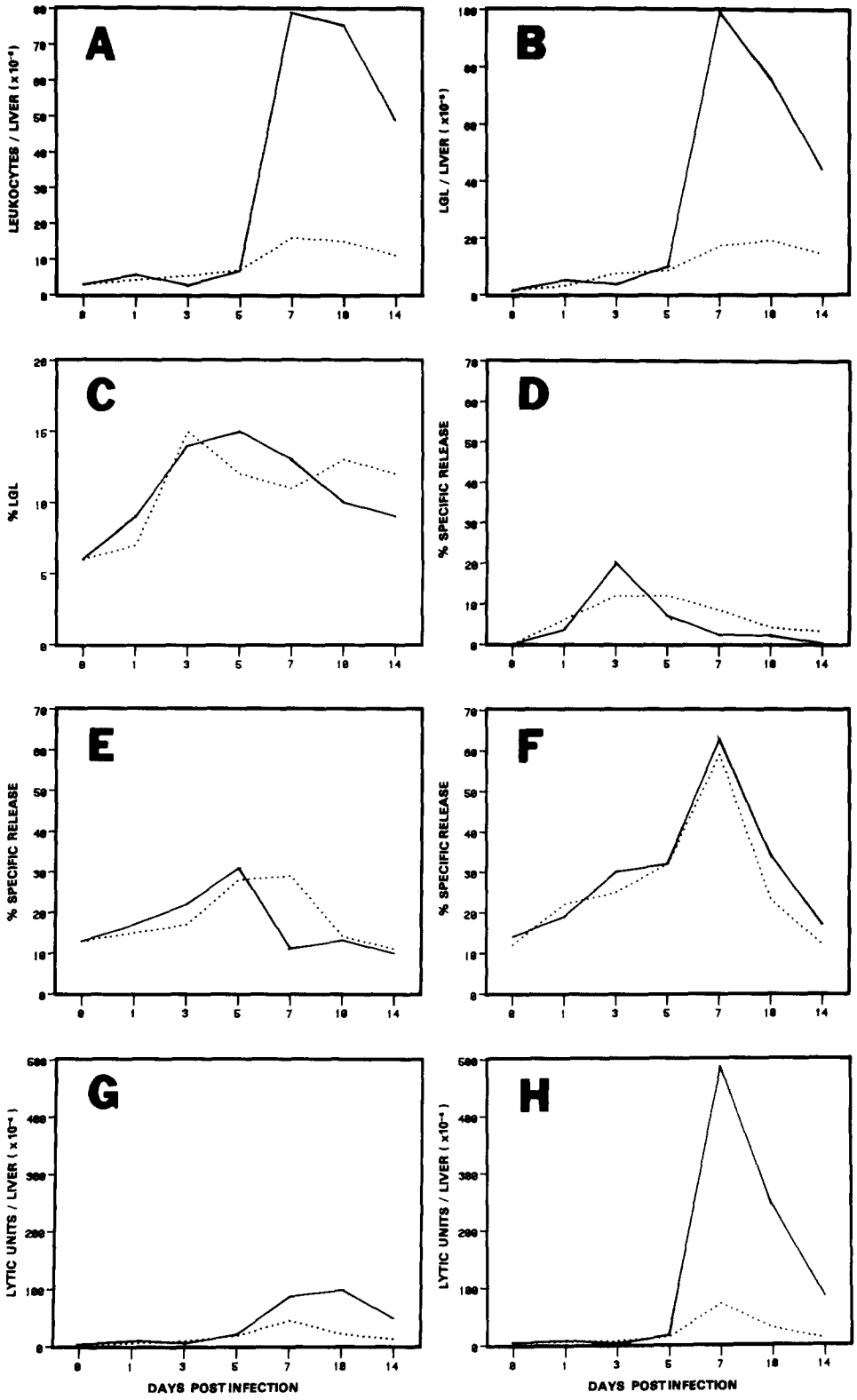
Cell Morphology. $1–3 \times 10^4$ of the nonparenchymal cells were centrifuged onto glass slides in a cytocentrifuge (Shandon Southern Instruments, Inc., Sewickley, PA) and stained with Wright's and Giemsa solutions. 200–500 cells were counted under oil immersion microscopy for each effector cell population studied. These preparations were >95% leukocytes by morphologic criteria.

Cytotoxicity Assay. Target cells (10^6) were labeled with 100 μCi ^{51}Cr (New England Nuclear, Boston, MA) for 1 h 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. NP-40 (1%) was added for maximum release determinations. The plates were incubated for 4–12 h 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, Inc., Palo Alto, CA). The coefficient of variation among quadruplicate wells was <5%. Data were expressed as percent specific ^{51}Cr release: 100 × (cpm experimental − cpm spontaneous)/(cpm maximum − cpm spontaneous). In some experiments that examined CTL activity, lysis was expressed as: percent virus-specific lysis = percent lysis of infected target − percent lysis of uninfected target. To compare the total lytic capabilities of effector populations isolated from different organs, calculations of total lytic units were computed by the formula: total lytic units = (fraction of targets lysed × number of cells per organ)/(E/T ratio). An E/T ratio on the linear portion of the cytotoxicity curve was used for these calculations.

Statistical Methods. Data are presented as mean ± SD. Student's *t* test was used for statistical comparisons between groups.

Results

Comparison of Hepatotropic and Nonhepatotropic LCMV Infections. The induction of NK cells and CTL activities and the accumulation of LGL in the liver were studied on days 1–14 p.i. in animals infected i.p. with either LCMV-ARM or LCMV-WE. LCMV-ARM is generally nonhepatotropic and grows poorly in



the liver during acute infection, whereas the LCMV-WE strain is hepatotropic and replicates in the liver to high titer (18). Both viruses induced an increase in NK cell activity detected against both YAC-1 and L929 cells by day 1 p.i. that peaked at days 3–5 p.i. and declined thereafter (Fig. 1 *D* and *E*). The appearance and progressive increase of NK cell activity through day 5 p.i. were paralleled by a progressive increase in LGL number beginning on day 1 p.i. (Fig. 1 *B*). Virus-specific CTL activity began to increase on day 5 p.i. with both viruses and peaked on day 7 p.i. (Fig. 1 *F*). By day 7 p.i., the LCMV-WE infection induced an average 28-fold increase in the number of liver leukocytes, whereas the LCMV-ARM infection induced only a fivefold increase (Fig. 1 *A*). These differences in total leukocyte number were confirmed histologically: LCMV-WE–infected livers had markedly greater infiltrations of mononuclear inflammatory cells than LCMV-ARM–infected livers (not shown). The centers of focal accumulations of mononuclear cells occasionally contained one or two necrotic hepatocytes with pyknotic nuclei and intensely eosinophilic cytoplasm. Because LCMV is relatively noncytopathic (28), it is likely that hepatocyte death results from an immunopathologic mechanism. When the total lytic capacities of the liver leukocyte populations were calculated, the LCMV-WE infection resulted in 10-fold higher total CTL lytic units on day 7 p.i. than did LCMV-ARM infection (Fig. 1 *H*). These levels of day 7 CTL activity closely correlated with the total numbers of LGL isolated on day 7 from the livers of mice infected with LCMV-WE or LCMV-ARM (Fig. 1 *B*).

Characterization of Day 3 and 7 Cytotoxic Effector Cells. The patterns of cytotoxic activities present at days 3 and 7 p.i. (Fig. 1) were suggestive of the NK cell and CTL lytic profiles reported previously in the spleen (8). To characterize these effector cell populations further, liver leukocytes were treated with various antibodies and complement (C) in vitro. The cells remaining after treatment were tested for cytotoxic activity and were examined for the presence of LGL on stained cytocentrifuge smears. The data in Table I (experiment 1) indicate that day 3 cytotoxic activity against YAC-1 cells was completely sensitive to treatment with anti–asialo GM_1 + C and slightly reduced by anti-Thy-1.2 + C. Treatment with C and J11d antibody (recognizing a marker on granulocytes and B cells [25]) had no effect on lysis. Changes in the number of cells morphologically identified as LGL paralled the changes in lytic activity: anti–asialo GM_1 + C treatment markedly reduced the number of LGL, anti-Thy-1.2 + C resulted in a partial depletion (64%), and J11d + C was without effect on LGL number. Thus, the phenotype for the day 3 p.i. effector cells is consistent with the reported phenotype of NK cells (29): asialo GM_1–positive, Thy-1.2$^{+/-}$, J11d^{-}, YAC-1 killer, and LGL morphology.

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

TABLE I

Characterization of Liver Leukocytes Isolated at Days 3 and 7 p.i. with LCMV-ARM

Exp.	Treatment	Percent Specific lysis at E/T ratios of:		Percent reduction in LGL ($\times 10^5$)
		20:1	7:1	
1. Day 3 effectors/YAC-1 targets	GpC control	29	12	1.4
	Anti-asialo GM + GpC	1.0	0.2	0.1 (93)
	Anti-Thy-1.2 + GpC	22	10	0.5 (64)
	RbC control	35	15	1.0
	J11d + RbC	34	15	1.1 (−10)
		7:1	2:1	
2. Day 7 effectors/percent virus-specific lysis	GpC control	30	17	15
	Anti-Thy-1.2 + GpC	8.8	0.7	4.5 (70)
	RbC control	32	15	8.4
	Anti-Lyt-2 + RbC	12	2.3	5.5

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

TABLE II

FACS Purification of Lyt-2$^+$ CTL/LGL at Day 7 p.i. with LCMV-ARM

Cell population	Percent LGL	Percent virus-specific lysis	Percent YAG-1 lysis
Lyt-2$^+$	42	57	3.1
Lyt-2$^-$	13	10	16
Unsorted	23	27	6.9

Liver leukocytes from C3H/St mice at day 7 p.i. with LCMV-ARM were stained with monoclonal anti-Lyt-2 antibody and sorted into Lyt-2$^+$ and Lyt-2$^-$ populations. NK cell assays against YAC-1 targets were run for 4 h at E/T of 3:1, and CTL assays against uninfected and LCMV-infected L929 cells were run for 14 h at E/T of 3:1.

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

To further confirm the CTL surface phenotype and LGL morphology of effector cells obtained at day 7 p.i. with LCMV, liver leukocytes were examined by FACS analysis after staining with monoclonal anti-Lyt-2 antibody. 27% of the day 7 p.i. liver leukocyte population was Lyt-2$^+$ (data not shown). FACS-sorted Lyt-2$^+$ cells were enriched at least twofold for virus-specific lytic activity compared with stained but unseparated cells, whereas NK cell activity was reduced (Table II). Conversely, while Lyt-2$^-$ cells were depleted of virus-specific cytotoxic effectors, they were enriched for cells expressing NK cell activity. Lyt-2$^+$ cells

TABLE III
Cytotoxicity Pattern of Day 7 p.i. Effector Cells from LCMV-ARM–infected Liver

E:T ratio	Percent specific lysis			
	L929	L929 + LCMV	L929 + PV	MC57G + LCMV
20	13	59	16	18
7	4.3	37	5.0	4.7
2	2.4	20	−0.6	4.7

Liver leukocytes from C3H/St mice (H-2^k) at day 7 p.i. with LCMV-ARM were tested for lytic activity against uninfected L929 cells (H-2^k), L929 cells infected with LCMV (L929 + LCMV) or Pichinde virus (L929 + PV), and MC57G cells (H-2^b) infected with LCMV (MC57G + LCMV) in 12-h ^{51}Cr-release assays.

were enriched almost twofold for cells possessing LGL morphology, and they accounted for 54% of all LGL present. Lyt-2^- cells were LGL-depleted compared with unfractionated cells (Table II). Thus, CTL activity and cells bearing LGL morphology were found to copurify in a population of leukocytes expressing the CTL marker Lyt-2.

Spleen CTL induced during LCMV infection are virus-specific and H-2-restricted effector cells (12). When tested for lysis against various targets, liver effector cells isolated from C3H/St mice at day 7 p.i. had typical CTL-killing patterns (Table III). These effectors strongly lysed LCMV-infected histocompatible cells (L929 + LCMV) but not LCMV-infected histoincompatible cells (MC57G + LCMV). They also exhibited minimal lysis against histocompatible cells either uninfected or infected with Pichinde virus (L929 + PV). Other experiments showed that the PV-infected cells were highly sensitive targets for PV-immune CTL (30). These experiments confirm that the effector cells isolated from liver at day 7 p.i. with LCMV contain classical virus-specific CTL.

Depletion of LGL In Vivo. Mice were treated in vivo with anti–asialo GM_1 at the time of virus infection, and liver leukocytes and splenocytes were isolated at day 3 p.i. Table IV (exp. 1) demonstrates that, in vivo, anti–asialo GM_1 treatment was highly effective at depleting NK cell activity (YAC-1 lysis) in both the liver and spleen during virus infection. This treatment also significantly reduced both the percentage and absolute number of LGL in the liver leukocyte population without reducing the total number of liver leukocytes present in these animals. The sensitivity of the generation of the day 7 effector cells was similarly tested. In contrast to the day 3 NK/LGL, the accumulation of day 7 CTL/LGL in the liver was relatively resistant to a double treatment in vivo with anti–asialo GM_1 serum (Table IV, exp. 2). This treatment eliminated most of the relatively low level of NK-like cytotoxicity against YAC-1 cells without reducing the CTL-like cytotoxicity on LCMV-infected L929 cells.

LGL Response in C57BL/6J Mice. To be certain that the differential in the liver-associated CTL response during LCMV-WE infection vs. LCMV-ARM infection was not peculiar to the C3H/St mouse strain, C57BL/6J mice were infected with LCMV-WE or LCMV-ARM, and the CTL responses were compared. Livers of C57BL/6J mice infected with LCMV-WE had significantly

TABLE IV

Effects of Anti-asialo GM_1 Treatment In Vivo on Accumulation of NK/LGL and CTL/LGL in Liver during LCMV-WE Hepatitis

Exp.	Anti-asialo GM_1	Percent YAC-1 lysis*		Total leukocytes per liver ($\times 10^{-6}$)	Percent LGL	Total LGL per liver ($\times 10^{-5}$)	Percent virus-specific lysis‡	
		Liver	Spleen				Liver	Spleen
1. Day 3	−	43 ± 11	36 ± 8.3	1.7 ± 0.1	25 ± 1.1	4.4 ± 0.3	ND	ND
	+	1.6 ± 0.6	4.3 ± 0.8	2.0 ± 0.6	5 ± 1	1.0 ± 0.3	ND	ND
2. Day 7	−	15 ± 3.1	11 ± 3.8	60 ± 8.1	14 ± 3.8	86 ± 24	41 ± 4.4	38 ± 10
	+	6.3 ± 0.6	4.3 ± 0.1	59 ± 3.0	12 ± 2	71 ± 16	50 ± 6.1	40 ± 7

Splenocytes and liver leukocytes were obtained from C3H/St mice ($n = 3$ per group) at day 3 p.i. (exp. 1) and at day 7 p.i. (exp. 2) with LCMV-WE. Antiserum-treated mice were injected i.v. with 0.2 ml anti-asialo GM_1 (1:10 dilution) at the time of infection (day 0; exp. 1) or at days 0 and 4 p.i. (exp. 2).

* YAC-1 assays were run for 4 h at liver leukocyte E/T ratio of 30 and spleen E/T ratio of 100.

‡ Percent virus-specific lysis was determined in 16-h ^{51}Cr-release assays at liver leukocyte E/T ratio of 3 and spleen E/T ratio of 11.

TABLE V

CTL Activity in Livers But Not in Spleens of C57BL/6J Mice Infected with LCMV-WE

Effectors	Total leukocytes per:		Percent LGL (liver)	Total LGL per liver ($\times 10^{-5}$)	Percent virus-specific lysis		Total CTL lytic units ($\times 10^4$)	
	Liver ($\times 10^{-6}$)	Spleen ($\times 10^{-7}$)			Liver	Spleen	Liver	Spleen
Control	10 ± 2.8	4.7 ± 1	4.0 ± 1.4	3.8 ± 0.3	—	—	—	—
LCMV-ARM	17 ± 1.4	11 ± 0.7	18 ± 5.7	30 ± 7.1	48 ± 9.9	54 ± 13	55 ± 9.9	114 ± 36
LCMV-WE	63 ± 21	3.6 ± 1	12 ± 4.2	71 ± 1.3	25 ± 0.7	13 ± 0.7	108 ± 4.1	8.8 ± 1.8

Liver and spleen effector cells were isolated at day 7 p.i. Lytic unit calculations are based upon percent virus-specific lysis determinations from 5-h ^{51}Cr-release assays at liver leukocyte E/T ratio of 20 and spleen E/T ratio of 50.

higher numbers of total leukocytes ($p < 0.02$) and total LGL ($p < 0.001$), as well as twofold higher CTL lytic units than did those from LCMV-ARM-infected mice (Table V). These results were similar to those observed with C3H/St mice (Fig. 1). Surprising, however, was the finding that this difference in the liver CTL/LGL activity was manifested despite the very low levels of CTL activity in the spleens of the LCMV-WE-infected mice (Table V). The spleens from the LCMV-WE-infected mice were smaller and contained 67% fewer leukocytes than spleens from LCMV-ARM-infected mice. Thus, C57BL/6J mice infected with either strain of LCMV were capable of mounting a CTL response, but that response was concentrated almost exclusively in the livers of mice infected with hepatotropic LCMV-WE, whereas the CTL response predominated in the spleens of mice infected with nonhepatotropic LCMV-ARM.

Thymic Dependence of LGL Accumulation. The reason for the large increase in total liver leukocytes after LCMV-WE infection as compared with LCMV-ARM infection (Fig. 1, Table V) was unclear. To determine whether specific T cell–mediated immunity played a role in this difference, T cell–deficient BALB/c *nu/nu* mice and immunocompetent *nu/+* littermates were infected with either LCMV-ARM or LCMV-WE, and liver leukocytes were isolated at day 7 p.i. The data in Table VI indicate that the T cell–sufficient *nu/+* mice infected with

TABLE VI

Inflammatory Cell Responses in Liver at Day 7 p.i. with LCMV-WE Are Thymus Dependent

Mouse strain	Virus strain	Total leukocytes per liver ($\times 10^{-6}$)
nu/nu	LCMV-ARM	8.8 ± 1.9
nu/+	LCMV-ARM	12 ± 2.3
nu/nu	LCMV-WE	7.9 ± 1
nu/+	LCMV-WE	37 ± 5.8

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

TABLE VII

Comparison of Augmentation of Liver NK/LGL by Poly(I-C), LCMV-ARM, MCMV, and MHV

Exp.	Strain	Treatment	Percent YAC-1 lysis	Total leukocytes per liver ($\times 10^{-6}$)	Percent LGL	Total LGL per liver ($\times 10^{-5}$)
1	C57BL/6J	Control	9.3 ± 2.3	3.6 ± 0.4	9.0 ± 1.4	3.2 ± 0.2
		Poly(I-C)	31 ± 7.1	3.0 ± 0.8	17 ± 0	5.0 ± 1.3
		Poly(I-C) + anti-asialo GM_1	1.0 ± 0.4	2.5 ± 0.6	1.5 ± 0.7	0.4 ± 0.3
2	C57BL/6J	Control	7.5 ± 3.1	7.9 ± 0.1	4.0 ± 1	4.0 ± 1.4
		LCMV-ARM	16 ± 0.6	12 ± 1.7	4.3 ± 0.6	5.2 ± 0.7
3	C67BL/6J	LCMV-ARM	16 ± 4.6	9.2 ± 2.6	9.0 ± 2.6	7.9 ± 0.8
		LCMV-ARM + anti-asialo GM_1	1.8 ± 2	9.9 ± 7.3	2.2 ± 1.3	2.2 ± 1.3
4	C57BL/6J bg/+	LCMV-ARM	15 ± 4	8.9 ± 3.5	8.3 ± 1.2	7.2 ± 1.7
	bg/bg	LCMV-ARM	2.3 ± 3	13 ± 2.3	11 ± 2.6	14 ± 3.5
5	C57BL/6J	Control	14 ± 0.7	6.7 ± 0.6	4.5 ± 0.7	3.0 ± 0.3
		MCMV	60 ± 4.9	14 ± 0.2	15 ± 1.4	21 ± 2.3
6	C57BL/6J	Control	3.5 ± 2.8	5.1 ± 0.9	3.0 ± 0	1.5 ± 0.3
		MHV	73 ± 4.2	26 ± 9.3	11 ± 2.8	33 ± 5
7	C57BL/6J	Control	10 ± 2.2	2.6 ± 0.8	4.5 ± 1	1.2 ± 0.3
		MHV	48 ± 3.6	9.5 ± 2.8	12 ± 3	12 ± 5.6
		MHV + anti-asialo GM_1	4.2 ± 4.9	4.3 ± 0.8	2.3 ± 0.6	2.2 ± 0.7
8	C57BL/6J bg/bg	Control	0.6 ± 0.8	13 ± 2.9	6.5 ± 1.7	8.4 ± 3.2
	bg/bg	MHV	9.3 ± 3.5	34 ± 5.1	13 ± 1.5	45 ± 11
9	BALB/cJ	Control	14 ± 3.8	14 ± 3.8	3.0 ± 1	4.0 ± 1.4
		LCMV-ARM	39 ± 13	23 ± 9.5	5.7 ± 1.5	12 ± 3.3
10	BALB/c *nu/nu*	Control	3.0 ± 2.1	5.6 ± 1.1	6.0 ± 1.4	3.5 ± 1.5
	nu/nu	Poly(I-C)	38 ± 4.5	5.2 ± 1.1	17 ± 2.5	9.9 ± 3
	nu/nu	LCMV-ARM	44 ± 5	6.5 ± 1.1	16 ± 3.1	11 ± 3.9
11	BALB/c *nu/+*	Poly(I-C)	36 ± 2	7.5 ± 0.7	16 ± 3	12 ± 2.7
	nu/nu	Poly(I-C)	55 ± 5.1	5.0 ± 0.7	27 ± 3.6	14 ± 3.8

LCMV-WE accumulated more liver leukocytes than did *nu/+* mice infected with LCMV-ARM ($p < 0.01$). This appeared to be thymus-, and therefore probably T cell–dependent, as *nu/nu* mice infected with either LCMV-WE or LCMV-ARM had lower numbers of liver leukocytes than *nu/+* mice infected with LCMV-WE.

Augmentation of Liver NK/LGL by Other Virus Infections or Poly(I-C). The effects of poly(I-C) or infection by several viruses on liver NK/LGL were compared in different mouse strains (Table VII). C57BL/6J mice treated with poly I:C (exp. 1) or infected with the noncytopathic LCMV-ARM (exp. 2) had comparable increases (two- to threefold compared with controls) in total LGL and in lysis of

YAC-1 cells. In vivo treatment with anti-asialo GM_1 serum abrogated NK cell activity and LGL accumulation in both cases (exps. 1 and 3). However, total numbers of liver leukocytes in the treated animals were only minimally changed from control, whether or not the mice received anti-asialo GM_1 injections.

NK cell-deficient C57BL/6J mice homozygous for the beige mutation (*bg/bg*) were compared with NK cell-sufficient *bg/+* littermates at day 3 p.i. with LCMV-ARM for liver NK/LGL lytic activity and cell number (exp. 4). C57BL/6J *bg/bg* mice had markedly reduced NK cell activity associated with liver leukocytes, despite having higher percentages and higher total numbers of LGL than *bg/+* mice. However, the majority (76%) of the liver LGL present in the *bg/bg* mice bore the atypical morphology characteristic of LGL from beige mutant mice (31), i.e., one to three abnormally large cytoplasmic granules rather than the numerous small granules of normal LGL (morphology not shown). The NK cell activity and accumulation of NK/LGL in the livers of heterozygous *bg/+* mice (exp. 4) were similar to those seen in C57BL/6J (+/+) mice (exps. 2 and 3).

These results showing only modest increases in total leukocyte and LGL number during LCMV infection contrast with the findings at day 3 p.i. in C57BL/6J mice infected with the cytopathic hepatotropic viruses, MCMV (exp. 5) or MHV (exp. 6). These virus infections increased the total leukocyte number two- to fivefold, LGL percentages three- to fourfold, and the total LGL number from 7- to 22-fold. NK cell activity (YAC-1 lysis) was also markedly increased in the MCMV- and MHV-infected mice. As also shown above for poly(I-C) and LCMV-ARM, anti-asialo GM_1 treatment abolished the NK/LGL increases induced by MHV (exp. 7). MHV infection of C57BL/6J *bg/bg* mice also resulted in a significant ($p < 0.01$) increase in NK/LGL in the liver, but these cells were relatively inefficient in lysing YAC-1 targets (exp. 8).

NK/LGL responses in normal BALB/cJ and in T cell-deficient nude (*nu/nu*) BALB/c mice were examined. After LCMV-ARM infection, BALB/cJ mice (exp. 9) were found to produce NK/LGL responses comparable to those seen in either C57BL/6J or C3H/St mice. In BALB/c *nu/nu* mice, poly(I-C) treatment and LCMV-ARM infection induced similar increases (exp. 10). When *nu/nu* and *nu/+* mice were compared within the same experiment, poly(I-C) treatment induced higher YAC-1 lysis and higher percent LGL in *nu/nu* than in *nu/+* mice (exp. 11). This is in agreement with previous observations of higher levels of resting NK cell activity in *nu/nu* than in *nu/+* mice (32).

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

Discussion

This is the first demonstration of the accumulation of both NK/LGL and CTL/LGL in the liver during a virus infection. The accumulation of these cells correlates with the appearance of NK cell and CTL cytotoxic activities. During the early course of infection, cytopathic viruses induce more extensive NK/LGL responses in the liver than do noncytopathic viruses. Infection with the hepatotropic LCMV-WE strain results in a significantly greater CTL/LGL response in

the liver on day 7 p.i. than does infection with the nonhepatotropic LCMV-ARM strain.

The NK cell activity in the liver is similar to the NK cell activity reported in the spleen during LCMV infection (8). The phenotype of the liver-derived NK cells is asialo GM_1–positive, Thy-$1.2^{+/-}$, and $J11d^-$, and the cell is contained within a population of leukocytes bearing the LGL morphology (Table I). Other experiments showed that liver NK/LGL activated in vivo by poly(I-C) treatment or MHV infection could be enriched in plastic-nonadherent cell populations (data not shown). The early appearance (day 1–2 p.i.) of augmented NK cell lytic activity in the liver is also seen in the spleen (8), where activity peaks around day 3 p.i. and then declines. Furthermore, the liver leukocytes contain "activated" NK cells at day 3 p.i. as evidenced by their ability to lyse L929 cells, a target resistant to "endogenous" NK cell–mediated lysis (Fig. 1 *D*). The lysis of NK cell-sensitive YAC-1 cells by liver leukocytes continues to remain above endogenous levels on day 7 p.i. with LCMV-ARM (Fig. 1 *E*), but lysis of the more resistant L929 cells is declining (Fig. 1 *D*). This prolonged elevation of NK cell–mediated lysis against YAC-1 cells has also been observed in peripheral blood (33). The circulating NK cells at day 7 p.i. are lytically active against YAC-1 targets but they have lost their ability to lyse the more resistant L929 cells (33). Thus, the high NK cell–mediated lysis against YAC-1 cells seen in the liver and blood at day 7 p.i. is more likely due to the high NK cell number rather than to an activated state of the NK cell population. This may reflect the fact that IFN levels, required for NK cell activation, decline after 3 d p.i. (8).

The early cellular (NK/LGL) immune responses in the liver after nonhepatotropic LCMV-ARM and hepatotropic LCMV-WE infections are quite similar, with NK cell activities and LGL numbers being relatively parallel for the two infections through day 5 p.i. (Fig. 1). This may indicate that the augmentation of NK cell activity and number in the liver is due to systemic effects of IFN rather than to virus replication in the liver. The IFN-inducer poly(I-C) stimulates comparable increases in NK cell activity and number (Table VII). In contrast, MHV and MCMV, which cause highly cytopathic infections of hepatocytes, stimulate much higher levels of NK/LGL in the liver (Table VII). This is also apparent in beige mutant mice which, despite having defective NK cell function, respond to cytopathic MHV infection with large numbers of abnormal NK/LGL. In these circumstances, the presence of the virus, by virtue of its lysing target cells, may provide an important stimulus for NK cell accumulation. Bukowski et al. (4) demonstrated that NK cells play a role in the early resistance to MCMV infection, and suggested that NK cells may provide resistance against MHV infection as well (23). In contrast, LCMV infection does not seem to be regulated by NK cells (4, 23). The tendency of a particular virus infection to attract a significant number of NK/LGL to a site of virus replication may be one factor in determining its sensitivity to NK cell–mediated antiviral resistance.

Several lines of evidence support the notion that the CTL lytic activity of liver leukocytes is being mediated by cells with an LGL morphology: (*a*) the CTL activity and the LGL present in the liver on day 7 p.i. are mostly resistant to in vivo treatment with anti-asialo GM_1, a treatment that depletes NK/LGL (Table IV); (*b*) leukocytes isolated from livers at day 7 p.i. with LGL morphology and CTL activity are at least partially sensitive to in vitro treatments with anti-Thy-

1.2 + C and anti-Lyt-2 + C (Table I); (*c*) CTL activity and LGL at day 7 p.i. are enriched in FACS-sorted Lyt-2$^+$ leukocytes (Table II), and (*d*) the levels of CTL activity and numbers of LGL are very closely correlated on days 7–14 p.i. (Fig. 1). Thus, we suggest that virus-specific CTL in the liver at day 7 p.i. are LGL, and that the activity and number of these cells are directly related to the extent of virus replication that has occurred in this organ.

The basis for the large inflammatory cell response in the livers of mice infected with the hepatotropic LCMV-WE was likely to be due either to an enhanced nonspecific inflammatory cell response that, by chance, included a proportion of CTL/LGL, or to virus-specific T cells (CTL/LGL ?) homing to the LCMV-WE-infected liver and generating signals that attracted other inflammatory cells into the organ. The lack of a large inflammatory cell infiltrate in any strain of mouse at 3 d p.i. or in *nu/nu* mice at 7 d p.i. suggests that the T cell hypothesis may be correct (Table VI). Supporting this hypothesis is that inflammatory responses in the brain or footpad of LCMV-infected mice are dependent upon the presence of H-2K- or D-restricted T cells (34, 35). The fact that a higher inflammatory response in the liver is seen with LCMV-WE than with LCMV-ARM is probably due to the fact that LCMV-WE replicates to higher titers (>100-fold) in the liver. The LCMV-ARM infection does not appear to prevent accumulation of leukocytes in the liver, as mice coinfected with both LCMV-ARM and LCMV-WE accumulate liver leukocytes and LGL comparable with mice infected only with LCMV-WE (data not shown).

The majority of experimentation in murine viral immunology has relied on findings obtained predominantly from spleen cells. The data presented in Table V demonstrate that, under certain circumstances, cytotoxic activity mediated by spleen cells may not accurately reflect ongoing immune responses elsewhere in the body. The basis for the markedly reduced spleen CTL response in C57BL/6J mice infected with LCMV-WE (compared with similarly infected C3H/St) is not known. Two possibilities are that there is a defect in the spleen for the generation of LCMV-specific CTL under these conditions of LCMV infection, or else the liver may serve as a stimulus to rapidly recruit CTL out of the spleen. These findings underscore the importance of examining other compartments of the virus-infected host for signs of a localized immune response, especially if the virus exhibits specific tissue tropism(s).

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

Summary

The immunologic mechanisms involved in virus-induced hepatitis were examined by measuring the cytotoxic capabilities and the morphologic and antigenic phenotypes of leukocytes isolated from livers of virus-infected mice. Large granular lymphocytes (LGL) of both natural killer (NK) cell and cytotoxic T

lymphocyte (CTL) phenotypes were found to accumulate in livers of mice infected with either the nonhepatotropic Armstrong strain of lymphocytic choriomeningitis virus (LCMV-ARM) or the hepatotropic WE strain (LCMV-WE). Between days 1 and 5 postinfection (p.i.), both viruses induced a three- to fourfold increase in NK cell lytic activity in the livers of C3H/St mice and a three- to fourfold increase in the number of LGL in the organ. These LGL were characterized as NK cells on the basis of cell surface antigens, kinetics of appearance, target cell range, and morphology. By day 7 p.i., virus-specific, H-2-restricted, Thy-1^+, Lyt-2^+, CTL activity was present in the liver, and its appearance correlated with a second wave of LGL accumulation. CTL activity, total leukocyte number, and CTL/LGL number were at least fivefold higher in the livers of mice infected with LCMV-WE than with LCMV-ARM. The dramatic LCMV-WE-induced day 7 increases in total leukocytes and LGL were absent in athymic nude (*nu/nu*) mice, suggesting that the increases were T cell–dependent. LCMV-ARM infection of C57BL/6 mice induced significant spleen CTL activity but little liver CTL activity, whereas LCMV-WE infection resulted in significant liver CTL activity but minimal spleen CTL activity.

Mice infected with the cytopathic hepatotropic viruses, mouse hepatitis virus (MHV) and murine cytomegalovirus (MCMV), experienced much greater increases in liver NK/LGL by day 3 p.i. than did mice infected with LCMV or injected with the interferon-inducer poly(I-C). MHV-infected mice homozygous for the beige (*bg/bg*) mutation also exhibited significant increases in liver NK/LGL cell number and activity, although the activity was less than heterozygote controls, and the morphology of the LGL granules was aberrant.

These data show that the LGL accumulate in virus-infected organs, in this case, the liver. An early NK/LGL influx is most pronounced during infection with cytopathic hepatotropic viruses. This initial influx of NK/LGL is followed later by an influx of CTL also possessing LGL morphology. The CTL/LGL response in the liver is significantly greater during hepatotropic virus infections, even when a strong CTL response in the spleen is lacking.

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