T CELL–MEDIATED HEPATITIS IN MICE INFECTED WITH LYMPHOCYTIC CHORIOMENINGITIS VIRUS

Liver Cell Destruction by H-2 Class I–restricted Virus-specific Cytotoxic T Cells as a Physiological Correlate of the ^{51}Cr-release assay?

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When injected intracerebrally (i.c.),[1] lymphocytic choriomeningitis virus (LCMV) usually causes a lethal lymphocytic choriomeningitis (LCM) in mice (1, 2). This disease has been shown (1–4) to be T cell mediated; immunocompetent mice develop LCM and die, whereas T cell–deficient mice survive. Susceptibility of mice to LCM disease depends upon the degree of T cell immunocompetence of the host, and varies with host genes (5, 6) and with different virus isolates (1, 2, 7). Since i.c. infections do not usually occur in nature, except for possible routing via the olfactory nerves, the model has been criticized as being artificial and therefore of questionable relevance. Although LCMV injected i.v. or i.p. only occasionally kills mice, they show varying signs of clinical disease during the course of the infection (1, 2, 6, 8–10).

In this report, we analyze the hepatitis caused in mice by hepatotropic LCMV (1, 2, 9, 10), by monitoring liver histology and the serum concentration of enzymes signalling liver cell destruction (i.e., alanine aminotransferase [ALT], glutamate dehydrogenase [GLDH], and less specific aspartate aminotransferase [AST]), or liver cell repair (i.e., alkaline phosphatase [AP]). We show that the liver cell damage is T cell mediated, independent of B cells and antibodies, and correlates best with the cytotoxic T cell activity in livers. LCMV-specific Lyt-2$^+$, H-2K,D-restricted T cells that were cytolytic in vitro caused, upon adoptive transfer, a dose- and time-dependent linear increase in serum enzyme levels in vivo. These data represent a physiological in vivo correlate of the ^{51}Cr-release assay *in vitro*, and give direct evidence that antiviral effector T cells act cytolytically in vivo. Overall, this virus-triggered but T cell–mediated liver disease resembles the pathophysiology of acute hepatitis B virus infections in man (11–13), and therefore may serve as an animal model of its immunological pathogenesis.

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[1] *Abbreviations used in this paper:* ALT, alanine aminotransferase; AP, alkaline phosphatase; AST, aspartate aminotransferase; GLDH, glutamate dehydrogenase; i.c., intracerebral; i.f., into the footpad; LCM, lymphocytic choriomeningitis; LCMV, LCM virus; LU, lytic unit.

Materials and Methods

Mice. Mice of either sex were used at an age of 8–16 wk. Inbred C57BL/6 (H-2^b), C57BL/10 (H-2^b), B10.D2 (H-2^d), B10.BR (H-2^k), A/J (H-2^a), ATL (H-2^{t1}), CBA/J (H-2^k), DBA/2 (H-2^d), and BALB/c (H-2^d), as well as outbred ICR +/+, *nu*/+, and *nu/nu* mice were obtained from the Institut für Zuchthygiene, Tierspital, Zürich. Inbred B10.G (H-2^q), B10.AKM (H-2^m), DBA/1 (H-2^q), and AKR (H-2^k) were purchased from OLAC, Bichester, England; NMRI outbred mice were from Ivanovas, Kissleg, Federal Republic of Germany. C57BL/6 +/+ and *nu/nu* mice were a gift of the Institut für Medizinische Forschung, Füllinsdorf, Switzerland.

Virus. The LCMV isolate WE was originally obtained from Prof. F. Lehmann-Grube, Hamburg, Federal Republic of Germany, and was subsequently propagated on L929 cells (1). LCMV-Armstrong was obtained as high-titered stock from Dr. M. Buchmeier, Scripps Clinic and Research Foundation, La Jolla CA, (14). Virus dilutions were made in MEM containing 1–5% of heat-inactivated FCS. The LCMV was either titrated in vivo in footpads (6, 11) or in vitro on Vero cells (1).

Vaccinia virus (Lancy isolate) was purchased as a lyophilized stock from the Schweizerisches Serum und Impfinstitut, Bern, Switzerland, and was usually dissolved in medium with FCS to yield a stock solution containing 2×10^8 PFU/ml of virus.

Infection and Immunization. Mice were infected i.v. with 2×10^4–10^6 PFU of LCMV-WE or with 10^5 PFU of LCMV-Armstrong (4, 5, 16).

Serum Sampling. Mice were anesthetized with ether; blood was obtained from the retroorbital venous plexus using heparinized smooth-edged Natelson blood collecting tubes (Fisher Scientific Co., Pittsburgh, PA). In experiments where serum was collected at only one time, mice were ether anesthetized and bled by cutting the thoracic inferior vena cava and thereafter collecting blood from the thoracic cavity. The blood was transferred to Microtainer tubes (Becton Dickinson, Rutherford, NJ) and processed. Serum samples were analyzed individually or in pools of two to five donors.

Anti-IgM–treated Mice. Mice were injected i.p. with purified rabbit anti–mouse IgM serum within 24 h after birth. They received 0.1 ml on days 1, 2, and 3, and thereafter three times per week. The preparation of the antiserum, the housing of mice, as well as the monitoring of suppression was performed as detailed previously (15).

Histology. Organs were fixed in PBS with 4% formalin for 1–2 d. Sections were stained with H and E according to standard procedures.

Determination of Serum Enzyme Concentrations. AST (EC.2.6.1.1), and ALT (EC.2.6.1.2) were determined according to the procedure of the International Federation of Clinical Chemistry by a UV-absorbtion test (16, 17). GLDH (EC.1.4.1.3) was determined by a standard method as described (18). AP (EC.3.1.3.1) was determined according to Bowers and McComb (19) using *p*-nitrophenylphosphate as substrate. All reagents were purchased from Boehringer Mannheim, Mannheim, Federal Republic of Germany. A Hitachi 705 Selective analyser was used for the analyses (Hitachi Ltd., Nacka Works, Japan).

In some experiments, mice were bled and enzyme concentrations were determined in individual serum samples, and means ± SEM were determined. In other experiments, equally sized blood samples from several mice were pooled and analyzed. Values of means and SEM have been rounded up to the next 5 for values <50 U/liter, and to the next 10 for values >50 U/liter.

Cytotoxic T Cell Assay. The procedures have been described previously in detail (4, 5, 20). Briefly, single–spleen cell suspensions from C57BL/6 mice were incubated with 10^4 LCMV-infected or uninfected MC57G (H-2^b, methylcholanthrene-induced established cell line) or YAC (H-2^a, a natural killer cell–susceptible thymoma line) target cells at various ratios in round-bottomed 96-well plates (Petra Plastik; Inotech, Wohlen, Switzerland) for 5 h at 37°C in air supplemented with 5% CO_2. Specific ^{51}Cr-release from target cells was calculated by correcting for spontaneous ^{51}Cr-release; maximal lysis was determined in wells containing targets treated with 1 N HCl but no effector cells. Lytic units (LU), a measure of relative cytolytic activity, were determined according to established methods (20, 21) to be the number of lymphocytes necessary to lyse one-third of the

standard number of target cells (10^4 cells/well) during the standard test duration. LU per spleen or liver were computed by dividing the total number of lymphocytes per organ by LU (20, 21).

Isolation of Lymphocytes from Livers. All livers were processed individually and were kept on ice. Single-cell suspensions were made by gently pushing the liver through a stainless steel grid with a rubber-coated plastic syringe plunger. The resulting cell suspension was washed twice in medium and divided into two 12-ml aliquots. Each aliquot was underlayed with 10 ml of Ficoll-Hypaque solution (Seromed; Biochrom KG, Berlin, Federal Republic of Germany) in a 40-ml glass tube. The tubes were spun at 2,500 *g* for 20 min in a Centra-7 centrifuge. Cells retrieved from the interface were washed twice in medium, counted, and resuspended in the appropriate volume of medium for use in the cytotoxic T cell assay.

Adoptive Transfer Experiments. Recipient mice were sublethally irradiated with 650 rad and injected with 10^5–10^6 PFU of LCMV-WE i.v. 5 d before transfusion. Donor mice were infected either with 10^3 PFU of LCMV on day −8 or with 8×10^6 PFU of Vaccinia virus on day −6. Donor mice were killed, and single–spleen cell suspensions were prepared in balanced salt solution. Cell suspensions (1–2×10^8) were slowly transferred i.v. to recipient mice in 0.5 ml.

Antisera Plus C' Treatment. Culture supernatants of the following mAb-producing cell lines were used: rat anti–mouse Thy-1.2 (HO 13-4-9) (22), rat anti-Lyt-2 (HO 2.2) (23), and rat anti-L3T4 (GK 1.5) (24). Usually, 3 ml of hybridoma supernatant were incubated with 5×10^8 spleen cells for 30 min at $4°C$. The cells were then washed and subsequently incubated with 12 ml of Low-Tox rabbit serum (Cederlane Laboratories, Hornby, Ontario, Canada) at a dilution of 1:15 for 30 min at $37°C$ (25). Cells were treated twice. C' controls were incubated with medium instead of antibody.

Nylon Wool Separation of Lymphocytes. The procedure of Julius et al. (26) was used.

Determination of LCMV Titers in Livers. Livers were homogenized in glass tubes with Teflon pestles. The frozen and thawed samples were serially diluted 10-fold in medium before 30 μl aliquots were injected into two footpads. Footpad swelling on days 7–9 was recorded, and the greatest dilution still giving a positive reaction multiplied by the initial dilution factor was taken as the titer of infectious doses (ID) of LCMV.

Results

Clinical Symptoms in Mice after i.v. Infection with LCMV. Dependent upon the various parameters outlined in subsequent sections, young adult (6–12 wk of age), immunocompetent mice showed no sign of disease up to day 6 or 7. Between day 7 and day 10, mice infected with high doses of LCMV showed signs of general malaise; they moved slowly, were hunched, had ruffled fur and plepharitis. Occasionally mice died between day 12 and day 15, but overall mortality was low (~10%).

Measurements of Enzyme Levels in Serum after Infection of Mice with Different LCMV Isolates. AST, ALT, GLDH, and AP enzyme concentrations in the serum of outbred ICR and C57BL/6 mice were measured in individual mice on day 7 after infection with LCMV-WE or LCMV-Armstrong (Table I). Enzyme concentrations varied considerably from one mouse to the next in both infected groups, and the uninfected group of mice. The means of the values obtained, or the values determined in pools of serum samples from two to four mice (e.g., Tables II and III) usually showed great differences between infected and uninfected groups, dependent upon the enzyme measured and various other parameters. Mice infected with LCMV-Armstrong had AST, ALT, GLDH, and AP values that were within normal ranges or were only slightly increased. In contrast, mice infected with LCMV-WE showed serum enzyme values (means of values from

TABLE I

Comparison of Enzyme Values in Serum of Mice Infected i.v. for 7 d with 10⁵ pfu of LCMV-WE or LCMV-Armstrong

Mouse strain	LCMV	AST Measured values	AST Mean ± SEM*	ALT Measured values	ALT Mean ± SEM	GLDH Measured values	GLDH Mean ± SEM	AP Measured values	AP Mean ± SEM
ICR	WE	2,646		1,155		713		133	
		3,374	2,760 ± 320	1,204	1,120 ± 70	474	530 ± 90	70	110 ± 20
		2,261		987		400		119	
	Arm	133		63		41		70	
		238	180 ± 30	147	90 ± 30	67	45 ± 10	70	65 ± 5
		182		53		33		56	
	None	119		52		39		77	
		203	150 ± 30	35	40 ± 10	35	40 ± 5	56	80 ± 10
		126		35		49		98	
C57BL/6	WE	861		658		205		84	
		2,737	1,670 ± 560	1,911	1,200 ± 370	625	390 ± 130	140	110 ± 20
		1,428		1,043		352		112	
	Arm	119		35		32		119	
		147	140 ± 20	42	45 ± 10	34	35 ± 5	119	120 ± 2
		168		56		45		126	
	None	98		21		30		322	
		140	110 ± 15	35	40 ± 10	37	35 ± 5	112	200 ± 60
		105		49		32		168	

* Means and SEM were determined; means and SEM were rounded up to the next 10 (for values >50) or the next 5 (for values <50).

pooled sera) that were 10–15 times greater than normal for AST, 10–25 times greater for ALT, and 10–12 times higher for GLDH. AP values were not different from controls on day 7. These increased values signalled severe liver cell damage triggered by LCMV-WE.

Dose Dependence. Changes in serum enzyme concentrations depended upon the dose of LCMV-WE injected i.v. (Fig. 1). The increase of serum enzyme levels correlated positively with the increased amount of LCMV injected; with doses <10³ PFU, increases of serum values were small and very variable.

When doses >2 × 10⁵ PFU of LCMV-WE were used for infection, enzyme concentrations did not reach higher levels, but in contrast to when doses <2 × 10⁵ PFU were used, they tended to stay elevated up to days 13–16.

Dependence upon Route of Infection. Outbred ICR and inbred C57BL/6 mice were infected with 2 × 10⁵ PFU of LCMV-WE into the footpad (i.f.) (30 µl), i.c. (30 µl), i.v. (200 µl), or i.p. (200 µl). Serum enzyme sample values were determined from blood collected on day 7 (data not shown). Whereas injection i.v. or i.p. caused the greatest increases in enzyme concentrations of AST, ALT, and GLDH (10–20-fold increases), infection i.f. triggered no or only marginal increases (i.e., 10–30%); after i.c. infection, enzyme levels reached intermediate levels (4–7-fold increases).

Mouse Strain–dependence. Different mouse strains varied with respect to changes in serum enzyme levels when analyzed 7 or 14 d after LCMV-WE infection (Fig. 2). Increases in concentration of ALT as well as AST and GLDH
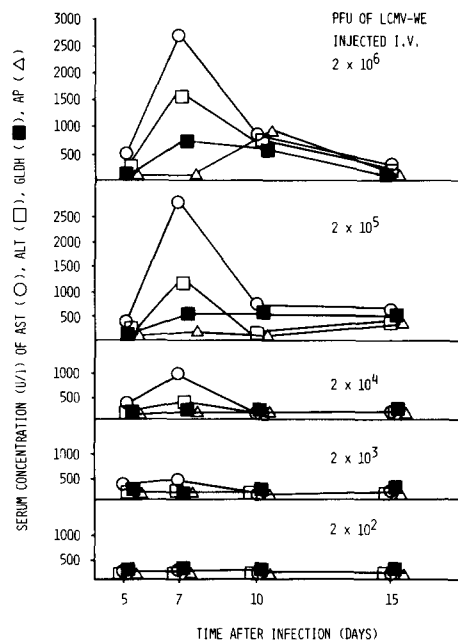
FIGURE 1. Kinetics and virus dose-dependence of the changes in enzyme concentrations in the serum of ICR mice. Mice were injected with the indicated doses of LCMV; two to four mice were bled at the times indicated, and the enzyme levels determined in the serum.
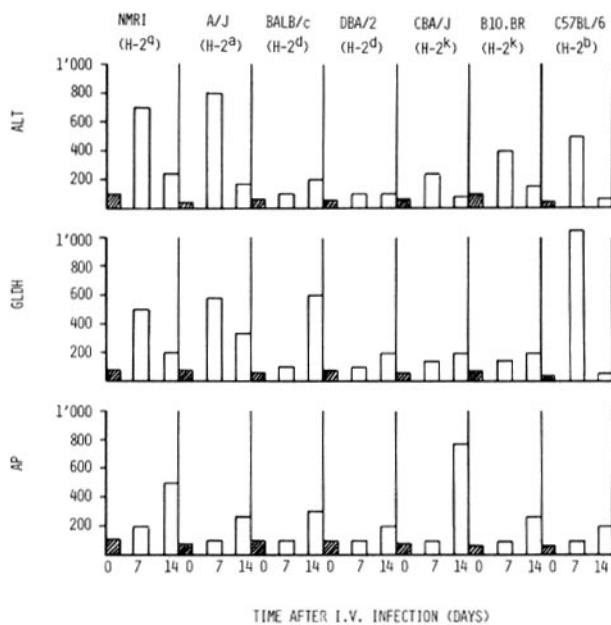

FIGURE 2. Mouse strain dependence of LCMV-triggered serum liver enzyme increases. Four mice of various outbred (NMRI) or inbred strains (all others) were infected i.v. with 10^5 PFU of LCMV-WE. On days 7 14, two mice were bled out and enzyme values were determined in pooled sera (□); control sera were pooled from two uninfected mice (▨).
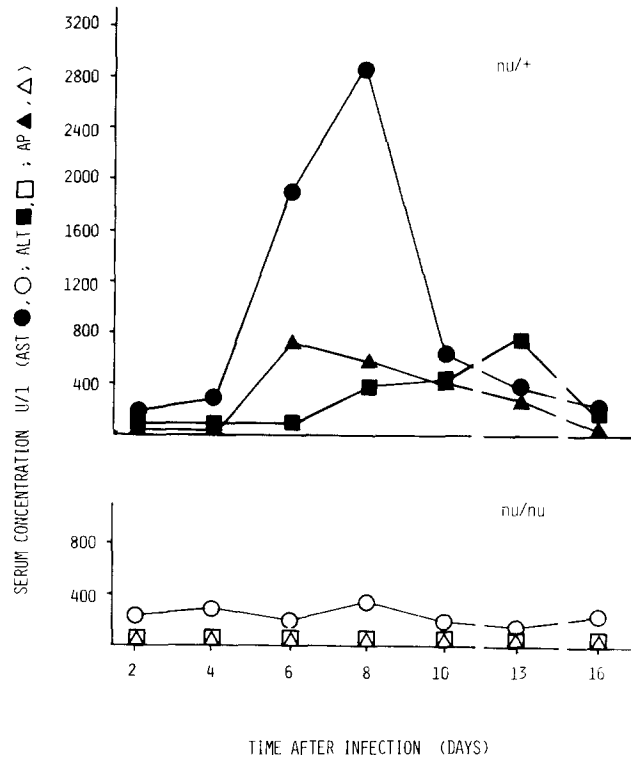
FIGURE 3.   Time course of hepatitis in immunocompetent and T cell–deficient mice. ICR *nu/+* (*top*) and ICR *nu/nu* (*bottom*) mice were infected i.v. with 2 × 10⁵ PFU of LCMV-WE; three to four mice were bled at each time point. Pooled sera were tested for AST (●, ○) ALT (▲, △) and AP (■, □).

were most pronounced in sera of outbred NMRI or ICR (not shown in Fig. 2, but shown in Figs. 1 and 3) and in inbred A/J, concentration increases were intermediate in those of inbred B10.BR and C57BL/6, and rather low in CBA/J, BALB/c, or DBA/2 mice. Significant increases in the serum enzyme levels of ALT and GLDH were seen on day 7, and were normally smaller or not significant on day 14 of infection; exceptions were BALB/c and DBA/2, which showed increased levels of these enzymes on day 14 when compared with day 7. AP values were usually higher on day 14 than on day 7, and were highest in CBA/J and NMRI mice. Thus with the same dose of LCMV-WE used to infect mice, enzyme concentrations varied widely in different mouse strains. There was no indication that the major histocompatibility gene complex H-2 influenced LCMV-WE-dependent enzyme changes.

T Cell Dependence of AST, ALT, and GLDH Increases, and of Liver Tissue Damage.   A detailed analysis of the time course of enzyme concentrations found in sera of outbred *nu/nu* and *nu/+* ICR mice (Fig. 3) confirmed similar findings (not shown) in C57BL/6 *nu/nu* and +/+ mice. ICR *nu/nu* mice infected i.v. with 2 × 10⁵ PFU of LCMV-WE showed no measurable changes in serum concentrations of the enzymes. In contrast, ICR *nu/+* mice exhibited dramatic increases of enzyme values by day 6; they peaked on day 6 for ALT, on day 8 for AST,
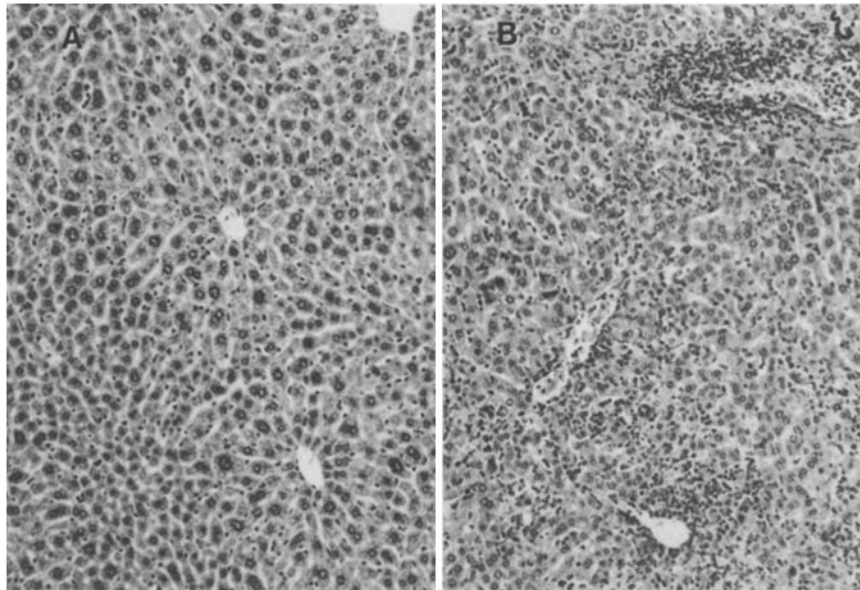
FIGURE 4.   Histology of livers from nu/nu ICR mice (A) or nu/+ mice (B) infected i.v. with ~2 × 10⁵ PFU of LCMV-WE 6 d previously. The sections are representative for the period of most severe disease.

and fell rapidly after 8 d. AP values rose after day 6 and peaked around day 13 in ICR nu/+.

The histological examination of liver sections from ICR nu/nu (Fig. 4a) revealed no signs of inflammatory reactions at any of the times evaluated (2, 4, 6, 8, 10, 13, and 16 d). In contrast, in nu/+ mice (Fig. 4b) periportal mononuclear infiltrates were associated with signs of focal liver cell destruction on days 6 and 8; also many lymphocytes were attached to the vascular endothelium. Similar sections from uninfected control nu/+ mice were comparable to the one shown from nu/nu mice. Histological signs of lymphocytic infiltrations and liver cell destruction were observed in nu/+ mice mainly from days 6–8; thereafter they decreased from days 10–13, and were absent on day 16.

Antibody-independence of Liver Damage.   The results obtained with nu/nu and nu/+ mice indicated that T cells were involved in causing both the histological alterations in livers and the changes in enzyme concentrations in sera of infected mice. A decisive contribution of T cell–dependent antibody responses was excluded by the results obtained with B cell–depleted mice. Chronically anti-IgM–treated mice were infected, shortly after birth, i.v. with LCMV-WE. Their serum enzyme levels are compared in Table II. In all mice, enzyme values were greatly increased when compared with uninfected controls, independent of whether they had been treated with rabbit anti–mouse IgM serum. However, after infection with LCMV-WE, anti-IgM–treated mice had greater AST, ALT, and GLDH concentrations in the serum than untreated (Table II, exp. 1) or normal rabbit serum–treated mice (Table II, exp. 2).

Correlation of Virus Titer and Serum Enzyme Values with Cytotoxic T Cell Activities in Liver.   Two to three C57BL/6 mice were infected i.v. with 2 × 10⁵ PFU of

TABLE II

Enzyme Values in Sera of LCMV-WE-infected B Cell–depleted Mice

Exp.	Mouse strain	Treatment of mice	Time after infection	Enzyme concentrations in serum			
				AST	ALT	GLDH	AP
			d	U/liter			
1	DBA/1	Anti-IgM*		1,120	130	ND	50
		Anti-IgM		1,540	360	100	20
		None	6	780	80	80	80
		None		720	100	ND	90
2	ICR	Anti-IgM‡		1,800	410	320	190
		Normal rabbit serum	9	1,380	260	100	110
	Control values of uninfected anti-IgM–treated mice			130–200	40–60	15–30	50–90

Mice had been treated with an affinity-purified rabbit anti–mouse IgM antiserum (see Materials and Methods), or were left untreated; DBA/1 were 14 wk old, ICR were 9 wk old at time of infection.
* Sera from individual mice.
‡ Serum pool from three mice.

LCMV-WE at various intervals before the assay; AST, ALT, and GLDH enzyme concentrations in the serum, appropriate virus titers per liver, and cytotoxic T cell activity in spleens were measured (Fig. 5). In addition, lymphocytes from the liver were isolated on a ficoll-hypaque gradient; the number of lymphocytes isolated and the cytotoxic activity found per liver paralleled all other parameters that were determined after day 6. On day 4, only virus titers in livers were high, but neither enzyme values in serum nor numbers of mononuclear cells that could be isolated by ficoll separation, nor cytotoxic T cell activity in livers were above background. Increases of enzyme concentration in serum and increases of cytolytic T cell activity in spleens and livers were roughly parallel between days 6 and 10. LCMV titers in livers changed minimally from days 6–10, but then decreased rapidly below detectable levels by day 12. In parallel with the disappearance of LCMV, both enzyme values and cytotoxic T cell activities fell to low or background values by days 13 and 16.

Thus the kinetics of effector T cell appearance and disappearance were delayed by ~3 d when compared with the growth curve of LCMV; once the virus was eliminated by day 13, LCMV-specific T cells decreased rapidly in livers and spleens and were not detectable any longer by day 16.

Cytotoxic T Cell Activity of Inflammatory Cells Isolated from Livers. When LCMV-specific cytotoxic activities were monitored in spleens and livers of C57BL/6 mice infected with 3×10^4 or 3×10^5 PFU of LCMV 8 d after infection, the dose responses shown in Fig. 6 were found. The effector cells were cytotoxic T cells because they lysed infected histocompatible (MC57G, H-2^b) but neither infected histoincompatible L929 (H-2^k), nor uninfected target cells, nor NK-susceptible YAC cells. In all examples tested, the relative activity of mononuclear
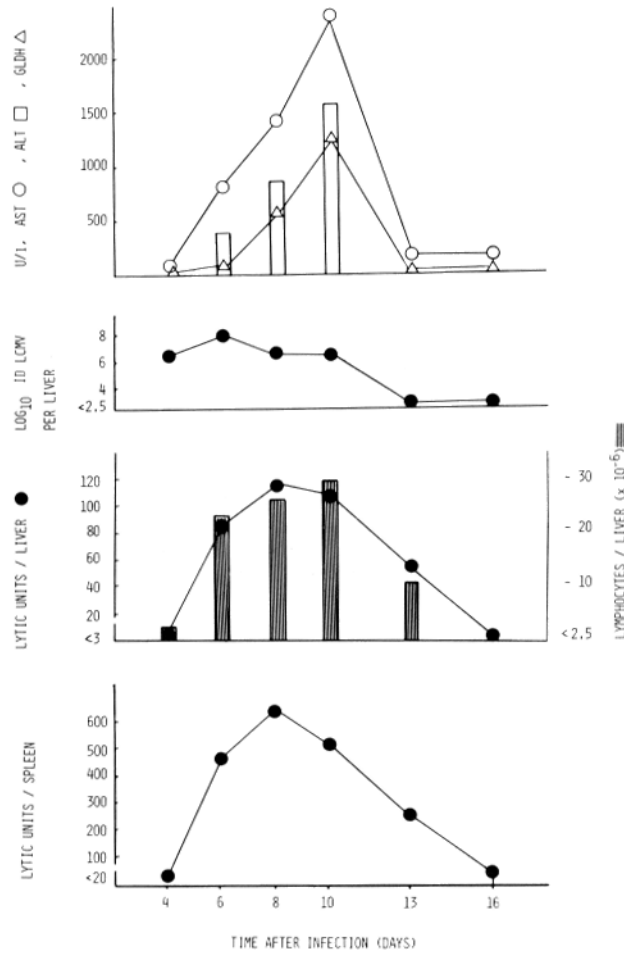
FIGURE 5. Comparison of the kinetics of LCMV in livers, liver enzyme concentration in serum and cytotoxic T cell activity in spleens and livers. C57BL/6 mice were infected with 2 × 10⁵ PFU of LCMV-WE i.v. at various times before the assay. Mice were bled out under anaesthesia; serum enzyme levels were determined as detailed in Materials and Methods (*top*). Virus titers in liver (*second panel*) number of liver lymphocytes recovered after ficoll separation and their cytolytic activity (*third panel*) and total lytic activity found in spleens were determined. All values are the average of three mice.

cells isolated from livers was, on a cell-for-cell basis, two to five times greater than that found in spleens from the same individual mice.

Adoptive Transfer of Liver Disease by T Cells. To evaluate which subpopulation of T cells was involved in pathogenesis of LCMV-triggered immune hepatitis, adoptive transfer experiments were performed. 8-d LCMV-WE–immune spleen cells (2 × 10⁸) from C57BL/6 mice were transferred to sublethally irradiated (650 rad at −5 d) and LCMV-WE (10⁶ PFU at −5 d)–infected recipient mice. The enzyme concentrations in serum were compared to values measured in identically pretreated mice that received 2 × 10⁸ 6-d Vaccinia virus–immune spleen cells. The difference between these two values was caused by LCMV-
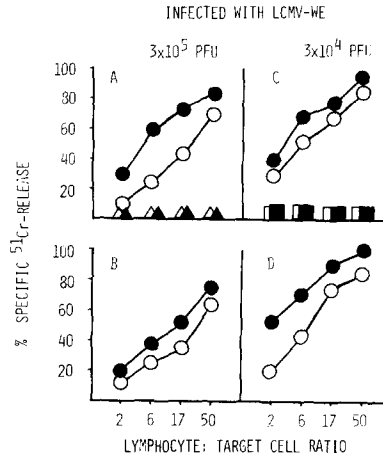
FIGURE 6. Cytotoxic T cell activity of mononuclear cells from livers and spleens. C57BL/6 mice (individuals A–D) were infected i.v. with the indicated dose of LCMV-WE, and 8 d later their lymphocytes from spleen (O) and livers (●) were assayed for cytotoxic activity on LCMV-infected MC57G. No specific ^{51}Cr release was measured on infected L929 (H-2^k) (△, ▲) or YAC (□, ■) target cells; also normal spleen cells did not lyse infected MC57G. Spontaneous release was <18% for all target cells used.
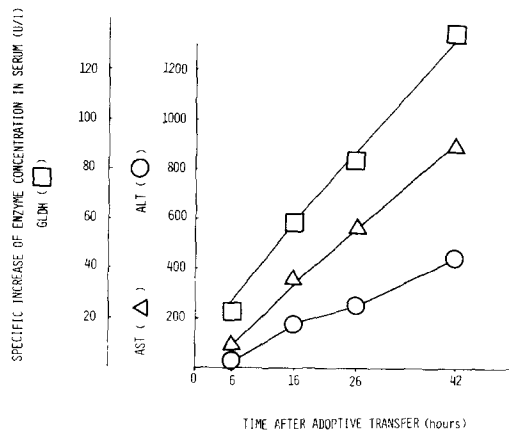

FIGURE 7. LCMV-immune or Vaccinia virus–immune spleen cells (2 × 10^8 in 0.5 ml) were injected i.v. into LCMV-WE infected (at −5 d) and irradiated (650 rad) recipient mice (C57BL/6). Mice were bled at the indicated times; specific increase of enzyme concentration was calculated as the difference between values obtained in recipients of LCMV-immune minus those of recipients of Vaccinia-immune spleen cells. Actual values (U/liter) for the 16-h time point were: for AST, (393 ± 38) - (68 ± 4); for ALT, (223 ± 62) - (28 ± 4); for GLDH, (66 ± 11) - (8 ± 1). Enzyme levels (U/liter) of unmanipulated control C57BL/6 mice were: AST, 60–100; ALT, 20–50; GLDH, 2–10.

specific T cells; the enzyme concentration values were found to increase in a linear mode dependent upon time (Fig. 7).

Cell Dose and Time Dependence of Serum Enzyme Level Changes. Nylon wool–nonadherent spleen cells were compared with unseparated spleen cells. 10^8 spleen cells transfused i.v. into preinfected recipients induced serum enzyme level increases comparable to those caused by 3 × 10^7 nylon wool–nonadherent

TABLE III
Characterization of Effector Lymphocytes

Exp.	Cells transferred		Recovery after treatment	Serum concentration of enzymes measured after transfer*					
				AST		ALT		GLDH	
	Treatment‡	Number		18 h	40 h	18 h	40 h	18 h	40 h
			%	U/liter					
A	1 None	None		200 ± 50	390 ± 90	60 ± 30	80 ± 30	25 ± 5	25 ± 5
	2 None	1 × 10⁸	100	1,950 ± 380	2,370 ± 340	1,530 ± 340	1,180 ± 530	410 ± 80	480 ± 160
	3 Nylon wool–	3 × 10⁷	30	1,830 ± 150	1,970 ± 170	1,450 ± 130	1,050 ± 130	420 ± 30	450 ± 30
	4 passed	1 × 10⁷		700 ± 140	1,070 ± 80	340 ± 140	430 ± 90	110 ± 40	130 ± 30
	5	3 × 10⁶		230 ± 70	590 ± 80	100 ± 10	200 ± 20	40 ± 5	60 ± 10
	6	1 × 10⁶		220 ± 60	500 ± 200	90 ± 40	100 ± 10	40 ± 10	50 ± 20
B	1 Anti-Thy-1.2 +	4 × 10⁷	40	250 ± 100§	ND	40 ± 5	ND	20 ± 10	ND
	2 C	4 × 10⁷	52	240 ± 10	ND	40 ± 5	ND	20 ± 10	ND
	3 Anti-Lyt-2.2 + C	4 × 10⁷	60	610 ± 50	ND	340 ± 50	ND	120 ± 20	ND
	4 Anti-L3T4 + C	4 × 10⁷	83	1,040 ± 140	ND	390 ± 160	ND	130 ± 50	ND
	5 C Control	None	100	200 ± 50		60 ± 30		25 ± 5	

* Donor mice were immunized with LCMV-WE (10⁵ PFU i.v.) 8 d previously. Recipient mice were irradiated (650 rad) and injected with 2 × 10⁶ PFU of LCMV-WE 5 d previously. Recipient mice were bled at the indicated time after cell transfer. Means ± SEM of three to five mice.
‡ See Materials and Methods.
§ Measured 18 h after transfer only. The same treated effector T cells were tested in vitro for their cytolytic activity at killer/target cell ratios of 17, 6, and 2:1 during a 5-h assay. Percent specific ⁵¹Cr release from infected target cells was: for cells treated with anti-Lyt-2.2 + C, 15, 6, 1; with anti-L3T4 + C, 75, 77, and 36; and with C alone, 72, 50, and 24. Spontaneous release was <25%; maximal release from uninfected targets <9%.

enriched T cells (Table III A). The dose- and time-dependent effect on serum enzyme levels by nylon wool–nonadherent lymphocytes is shown in Table I. Whereas 10⁷ T enriched spleen cells caused a significant increase of serum enzyme concentrations within 18 h, an augmentation of serum ALT and GLDH (but not of AST) caused by the transfer of 3 × 10⁶ cells could be assessed only after 40 h.

Characterization of Effector T Cells. The effector cells causing the increase in serum enzyme concentrations were identified as T cells by the following criteria: they were nylon wool nonadherent (Table III), and they were susceptible to treatment with anti-Thy-1.2 plus C and anti-Lyt-2 plus C, but resistant to anti-L3T4 plus C treatment (Table III). LCMV-specific cytotoxic activity of 8-d immune spleen cells treated with the various antibodies plus C was as expected; anti-Lyt-2 plus C treatment abrogated cytolysis by >95%, whereas anti-L3T4 plus C treatment enriched for cytolytic effector T cell function when compared with C treatment alone (Table III). Cytotoxic activity measured in vitro correlated with the capacity of the same cell populations to cause increases of serum enzyme concentrations. Anti-Lyt-2 plus C–treated cells failed to induce substantial increases, whereas anti-L3T4 plus C–treated cells caused ~10-fold increases of ALT and GLDH concentrations in serum of recipient mice.

Class I Restriction of Effector T Cells Mediating LCMV Hepatitis. The H-2 restriction of effector T cells causing liver cell destruction was assessed by adoptive transfer (Table IV) of 8-d immune spleen cells into irradiated (650 rad at −5 d) and preinfected (10⁶ PFU of LCMV-WE at −5 d) recipient mice.

To control for possible nonspecific effects on enzyme levels in LCMV-infected and irradiated recipients by transferred LCMV-immune spleen cells, enzyme

TABLE IV

Specific Changes of Serum Liver Enzyme Concentrations Caused by Adoptive Transfer of LCMV Immune vs. Vaccinia-immune Spleen Cells to LCMV-WE-infected Irradiated Recipient Mice

Group	Donor H-2 KID	Recipient H-2 KID	H-2 compatibility	Serum concentration (U/liter) of:					
				ALT; cells transferred:			GLDH; cells transferred:		
				LCMV-immune	Vaccinia-immune	Specific increase*	LCMV-immune	Vaccinia-immune	Specific increase
1	B10.AKM kkq	B10.AKM kkq	$K^k I^k D^q$	760 ± 430‡	40 ± 10	720	150 ± 70	20 ± 5	130
2		B10.G qqq	D^q	670 ± 110	60 ± 10	610	120 ± 20	20 ± 5	90
3		B10.BR kkk	$K^k I^k$	200 ± 100	80 ± 10	120	40 ± 10	25 ± 5	15
4		B10.D2 ddd	none	60 ± 20	70 ± 20	<1	20 ± 10	25 ± 5	<1
5		A/J kkdd	$K^k I^k$	1,100 ± 150	290 ± 140	810	480 ± 30	200 ± 100	280
6		A.TL skd	I^k	70 ± 10	180 ± 30	<1	80 ± 5	90 ± 10	<1
7	B10.D2 ddd	A/J kkdd	D^d	1,240 ± 80	280 ± 30	960	410 ± 20	90 ± 5	320
8	None	B10.AKM	—	50 ± 20	60 ± 20	<1	40 ± 20	40 ± 10	<1
9	None	B10.D2	—	60 ± 20	60 ± 10	<1	30 ± 10	25 ± 5	<1

* 2×10^8 immune spleen cells from donor mice infected i.v. with 10^3 PFU of LCMV-WE 8 d previously were transfused i.v. to recipient mice that had been pretreated with 650 rad (−5 d), and infected with LCMV (10^6 i.v., −5 d). Specific increase of enzyme levels in the serum was calculated by subtraction of enzyme values caused by adoptive transfer of 2×10^8 6-d Vaccinia-immune spleen cells from those caused by 2×10^8 8-d LCMV-immune spleen cells.

‡ Two to four mice per group were bled and individual sera were analyzed for enzyme concentrations; the numbers represent the average of each group ± SEM.

values were compared with those of mice transfused with Vaccinia virus–immune spleen cells. When serum enzyme concentration values in sera from recipients of Vaccinia-immune spleen cells were subtracted from the respective values found in sera from recipients of LCMV-immune spleen cells, we found that H-2 compatibility for H-2D alone caused great increases in both examples tested (Table IV, groups 2 and 7); recipients compatible only for H-2I did not show a specific increase (Table IV, group 6). H-2K-compatible effector spleen cells of B10.AKM origin caused a smaller increase in $K^k I^k$-compatible B10.BR recipients (Table IV, group 3) than the same effector cells caused in $K^k I A^k$-compatible A/J mice; this may reflect a considerably greater increase of serum enzyme levels caused by LCMV infection in immunocompetent A strain mice (Fig. 2) compared with B10 mice. Completely H-2-incompatible immune spleen cells did not cause drastic changes of serum concentrations when compared with control mice that did not receive immune cells (compare groups 4 and 9 in Table IV).

Discussion

LCMV-induced, immunopathologically mediated hepatitis depends upon specific viral and host parameters. The hepatotrope LCMV-WE isolate but not the neurotrope LCMV-Armstrong isolate causes hepatitis; usually, higher doses cause more severe disease. Infection i.v. or i.p. causes more severe disease than s.c. infection. The host parameters influencing hepatitis include the general genetic background of the mouse; A, ICR, and NMRI mice are most susceptible, C57BL and CBA intermediately susceptible, and BALB/c and DBA/2 mice least suscep-

tible to LCMV-WE hepatitis; however, no influence of the murine major histo-compatibility gene complex H-2 on susceptibility to LCMV-hepatitis has been found so far. A further crucial factor is the immunocompetence of the host, since euthymic mice do, while nude mice do not develop hepatitis; B cells and antibodies are not significantly involved, whereas activity of cytotoxic T cells parallels histological abnormalities in liver and changes of enzyme (AST, ALT, GLDH) concentrations in serum. Most symptoms of hepatitis fade in parallel with the clearance of virus and the rapid decrease of T cell activities. Mononuclear cells with cytotoxic T cell activity, but lacking NK activity, can be isolated from livers during the period of most florid hepatitis; when compared on a cell-for-cell basis, cytotoxic mononuclear cells from livers were relatively more active than spleen cells.

The presented results extend the classical studies on LCM disease describing clinical symptoms of systemic LCMV infections in mice, such as LCMV-induced wasting disease (1, 2) or hematological disorders (8). In nearly all instances, immune mechanisms mediated by T cells were shown to be responsible for the pathology; in few examples has a direct effect of LCMV on selected cellular functions been demonstrated (27). However, only in the case of LCMV-induced choriomeningitis have cytotoxic T cells been implicated directly (3, 4, 28, 44). Our analysis of LCMV-induced hepatitis illustrates that, here also, T cell–dependent and/or –mediated cell and tissue damage is responsible for disease rather than the virus itself. This is in contrast to other murine hepatitis forms caused by ectromelia virus (mousepox [29]) and probably also by murine hepatitis virus (MHV) (30) infections, which are mainly caused by the direct cytopathic effect of the virus itself. The model disease described here resembles in some aspects acute hepatitis B virus infections in humans (11–13), but differs in others. Hepatitis B virus and LCMV are poorly or noncytopathic (1, 2, 31), may be transmitted from mother to offspring (1, 2, 32) and may establish a virus carrier status in either newborn or adult individuals without causing clinical symptoms (11, 12). Also, in both infections, liver cell damage does not correlate with virus titers in liver or blood, but rather with the T cell immune response in mice and the presumably T cell–dependent IgG anti–hepatitis B surface antigen response in humans (11, 12, 50–54).

Over the past two decades, it has been repeatedly postulated that hepatitis B is immunologically mediated (26–28, 33–37). Several observations have supported this claim. Clinical symptoms usually become manifest at about the time when levels of hepatitis B surface antigens begin to fall, signalling successful virus elimination (12, 13, 34–37). Indirect evidence is provided by the finding that susceptibility to some forms of hepatitis B is weakly linked to major transplantation antigens (38). Furthermore, immunosuppression with lymphocytotoxic drugs may, dependent upon the particular equilibrium established in a patient between virus spread and immune response, temporarily improve signs of aggressive hepatitis (39) or alternatively, may induce or reactivate it (40). Also, transfer of HLA nonmatched hepatitis B–immune white blood cells to hepatitis B virus carriers has been shown in one study (41) to cause a rapid but only temporary increase of several liver enzymes in the blood of the recipient.

Cytotoxic virus-specific T cells have been searched for in the human disease but only with limited success so far. Possibly this may be because of at least three

reasons: hepatitis B virus is difficult to grow in tissue cultured cells in vitro (31, 42), the direct isolation of virus-specific cytotoxic T cells from peripheral blood is in general only rarely possible (43), and induction of secondary hepatitis B-specific cytotoxic T cells in vitro has not yet been achieved.

Obviously, the presented mouse model of T cell–mediated LCMV hepatitis differs in many other aspects from human hepatitis B infections. The incubation time in hepatitis B seems to be dose dependent and is usually long (weeks to months). After i.v. infection of LCMV, the incubation period of hepatitis in mice is 6–9 d, and this incubation time is influenced only marginally by the dose of virus used. Another clinically important feature of hepatitis B not found in LCMV hepatitis of ICR or C57BL/6 mice is the form of chronic, aggressive hepatitis; the established model of LCMV-hepatitis corresponds more to an acute form of hepatitis B. It will be of great interest to evaluate in this murine model both viral and host factors that may influence the balance between LCMV spread and immune response, and which may cause more chronic forms of hepatitis.

The present study also demonstrates as directly as possible that virus-specific T cells that are cytotoxic in vitro act cytolytically in vivo. When infected with the hepatotropic LCMV-WE isolate, a great number of host cells, including liver cells, are infected and therefore can serve as target cells in vivo for adoptively transferred LCMV-specific effector T cells. Since liver cells store a number of characteristic enzymes, which are released upon cytolysis, enzyme release could be used as an indication for cell death (44). Because of the potentially great number of available infected liver target cells, the resulting signal was great enough to be measurable within a relatively short time after adoptive transfer of cells to LCMV-infected immunosuppressed recipient mice. Adoptive transfer of nylon wool–nonadherent, Thy-1,$^+$ and Lyt-2,$^+$ L3T4,$^-$ class I– but not class II–MHC-restricted, and virus-specific cytotoxic T cells to irradiated and preinfected recipients led to a cell dose– and time-dependent linear increase of these enzymes in the serum. The linear time dependence of the serum enzyme changes after transfer suggests that effector T cells destroy infected liver cells in vivo in the same way as T cells lyse ^{51}Cr-labelled infected target cells in vitro (21). Both types of label releases follow a single-hit kinetics, signalling target cell destruction by cell contact rather than by soluble mediators (45–47).

The experimental design makes it improbable that lymphohemopoietic cells other than Lyt-2$^+$, L3T4$^-$ class I MHC–restricted T cells contributed significantly to the destruction of infected liver cells. Recipient mice had been irradiated with 650 rads, and therefore did not possess many lymphohemopoietic cells, including macrophages 5–6 d after irradiation. In addition, nylon wool–purified T cells that lacked great numbers of macrophages or B cells transferred hepatitis successfully in a class I–restricted fashion rapidly after transfer.

The question of how cytotoxic T cells mediate their effector function in vivo has been addressed repeatedly by analysis of the effect of purified or cloned cytotoxic T cells in vivo in various murine tumor and infectious disease models (20, 21, 28, 48, 49). Antiviral protection measured by virus titer reduction or prevention of titer increases may be interpreted to reflect direct host cell destruction during the eclipse phase of virus infection. This correlation was demonstrated in vitro by the finding that the ^{51}Cr-release from acutely infected target cells paralleled blocking of generation of virus progeny (20, 49). Tumor

cell destruction by cytotoxic T cells was shown by labelling tumor cells and then examining the excretion of the radioactive label (50). Cytotoxic T cells could be shown to cause rapid radiolabel excretion when both tumor cells and T cells were given i.p. When tumor cells were given i.p. and T cells i.v., label excretion was considerably slower and more difficult to measure accurately during the first 24–48 h, because of the background release of the label and because of the relatively smaller signal. Both of these technical problems have been considerably reduced in this study by using infected liver cells as target cells. In this physiological model, the background was low and stable, and many infected target cells were available and accessible to recirculating effector T cells.

Because of the experimental conditions chosen, the obtained results, particularly the time-dependent linear increase of liver enzyme levels in serum, represent the most direct evidence to date that cytotoxic effector T cells are directly responsible for enzyme release from LCMV-infected liver cells, and therefore that they act cytolytically in vivo.

Summary

A model for immunologically T cell–mediated hepatitis was established in mice infected with lymphocytic choriomeningitis virus (LCMV). The severity of hepatitis was monitored histologically and by determination of changes in serum levels of the enzymes alanine aminotransferase (ALT), aspartate aminotransferase (AST), glutamate dehydrogenase (GLDH), and alkaline phosphatase (AP). Kinetics of histological disease manifestations, increases of liver enzyme levels in the serum, and cytotoxic T cell activities in livers and spleens all correlated and were dependent upon several parameters: (a) LCMV-isolate; LCMV-WE caused extensive hepatitis, LCMV-Armstrong virtually none. (b) Virus dose. (c) Route of infection; i.v. or i.p. infection caused hepatitis, whereas infection into the footpad did not. (d) The general genetic background of the murine host; of the strains tested, Swiss mice and A-strain mice were more susceptible than C57BL or CBA mice; BALB/c and DBA/2 mice were least susceptible. (e) The degree of immunocompetence of the murine host; T cell deficient nu/nu mice never developed hepatitis, whereas $nu/+$ or $+/+$ mice always did. B cell–depleted anti-IgM–treated mice developed immune-mediated hepatitis comparably or even more extensively than control mice. (f) Local cytotoxic T cell activity; mononuclear cells isolated from livers during the period of overt hepatitis were two to five times more active than equal numbers of spleen cells.

Adoptive transfer of nylon wool–nonadherent anti-Thy-1.2 and anti-Lyt-2 plus C–sensitive, anti-L3T4 plus C–resistant lymphocytes into irradiated mice preinfected with LCMV-WE caused a rapid time- and dose-dependent linear increase of serum enzyme levels. This increase was caused by adoptive transfer of lymphocytes if immune cell donors and recipient mice shared class I, but not when they shared class II histocompatibility antigens. The donor cell dose-dependent increase of these enzymes was first measurable 6–18 h after transfer with 2×10^8 cells or 3×10^6 cells, respectively. The time-dependent increase caused by the adoptive transfer of $1–2 \times 10^8$ cells was strictly linear during a period of up to 25–40 h. These results indicate single-hit kinetics of liver cell death and suggest that effector T cells destroy infected liver cells via direct contact rather than via soluble toxic mediators. The results may represent the

best in vivo correlate of the in vitro ^{51}Cr-release assay that has been analyzed so far, and strongly support the view that antiviral cytotoxic T cells are directly cytolytic in vivo.

Thus LCMV-WE-induced hepatitis in mice is an immunopathologically mediated disease caused by T cell–mediated destruction of infected liver cells. Overall, this disease parallels many aspects of acute viral hepatitis in humans, which is caused by hepatitis B virus.

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