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Liver Damage by Infiltrating CD8\(^+\) T Cells Is Fas Dependent

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Ag stimulation of CD8\(^+\) lymphocytes in vivo results in their migration to various tissues as well as the activation of a cytolytic program involving perforin, TNF-\(\alpha\), and Fas ligand. The liver is one of the main sites for infiltration by activated CD8\(^+\) T cells, and this is followed by the death of hepatocytes. The contribution of the various cytolytic components to this process is unclear. Hepatocyte damage by CD8\(^+\) T cells was studied using the MHC class I-restricted OVA-specific TCR transgenic mouse (OT-1) to examine the contribution of Fas to hepatocyte death. Activated CD8\(^+\) T cells from both OT-1 and Fas-deficient OT-1\(lpr\) mice migrated to the liver in similar numbers after OVA administration, but only in OT-1 mice was there evidence of significant hepatocyte damage histologically and by elevation of serum aspartate transaminase. These differences were not the result of inefficient induction of cytolytic activity in OT-1\(lpr\) liver T cells, since they were as cytolytic in vitro as OT-1 liver T cells. This was supported by findings of similar high levels of message for perforin, TNF-\(\alpha\), and Fas ligand in infiltrating liver lymphocytes from both mice. These findings demonstrate that following Ag activation, infiltrating liver CD8\(^+\) T lymphocytes induce hepatocyte damage in a Fas-dependent manner. The Journal of Immunology, 2001, 167: 6654–6662.

A

citivation of lymphocytes in vivo leads to their migration to a variety of nonlymphoid sites, including the lung, intestine, and liver (1, 2). This results in part from the up-regulation of lymphocyte surface molecules important to trafficking, such as the hyaluronate receptor CD44, LFA-1, and various chemokine receptors (3).

The fate of lymphocytes once they have extravasated into tissues is less well understood, as are the consequences to the tissues that are infiltrated. The liver is a useful model system in which to examine these events, as it contains a resident population of T cells that expresses an activated phenotype, including expression of CD44 (4). Following Ag stimulation, the number of lymphocytes infiltrating the liver increases enormously (1). We have previously observed that the administration of Ag in vivo to TCR transgenic mice causes liver infiltration of Ag-specific CD4\(^+\) and CD8\(^+\) T cells, but only the CD8\(^+\) T cell subpopulation caused significant liver damage (5). Less apparent was the mechanism responsible for the liver damage.

Cytolytic T cells kill their targets in vitro primarily through perforin and Fas ligand (FasL)\(^\dagger\) (6, 7). In vivo this may also involve additional effector molecules such as TNF-\(\alpha\) (8, 9). Hepatocytes are exquisitely sensitive to Fas-induced death following in vivo administration of anti-Fas Ab (10). However, studies of lymphocyte-mediated liver injury have achieved various results regarding which pathways were critical for hepatocyte death. These differences depended somewhat on the lymphocyte activation method used. Studies using in vivo administration of Con A observed that hepatocytes were killed by either the perforin pathway (11) or required IFN-\(\gamma\) (12, 13), with little involvement of FasL or TNF-\(\alpha\). The findings in one study of a murine model of hepatitis B found little involvement of perforin or FasL in hepatocyte injury (14); however, conflicting data were reported by Kondo et al. (15), supporting a role for FasL. Still a third model used OVA-primed liposomes targeted to the liver and observed that adoptive transfer of OVA-specific Th1, but not Th2 CD4\(^+\) T cells induced liver injury that was mediated by IFN-\(\gamma\) and TNF-\(\alpha\), but not FasL (16). In none of these studies were liver lymphocytes actually purified and analyzed. It is thus unclear what was the actual level of cytolytic activity or expression of cytolytic mediators by the infiltrating T cells.

In the current studies, we have extended our earlier findings of hepatocyte injury mediated primarily by Ag-specific CD8\(^+\) T cells to further examine the specific method of cytology used by the liver-infiltrating T cells. For this purpose, we used the OT-1 mouse whose T cells recognize the OVA peptide (OVAp) (3), SIINFEKL, in the context of the MHC class I molecule H-2\(K\(^b\)\) (17). Fas-deficient OT-1\(lpr\) mice manifested markedly less hepatocyte damage despite similar CD8\(^+\) T cell activation, liver infiltration, and expression of cytolytic effector molecules. They also did not develop any accumulation of Ag-activated T cells in lymphoid tissues.

Materials and Methods

Mice

Strains of C57BL/6, C57BL/6\(lpr\), and transgenic OT-1 and OT-1\(lpr\) mice were bred at the animal facilities of the University of Vermont College of Medicine. Original breeding pairs of C57BL/6 mice were obtained from The Jackson Laboratory (Bar Harbor, ME). OT-1 mice bear a transgenic TCR that recognizes chicken OVAp 257–264 restricted to class I MHC, K\(^b\), and were provided by F. Carbone (Monash University Medical School, Victoria, Australia) and M. Bevan (University of Washington, Seattle, WA). OT-1 mice were maintained by breeding TCR transgenic males to normal C57BL/6 females. Offspring were screened for the clonotype TCR using anti-V\(\alpha\)2 mAb. Breeding OT-1 mice with C57BL/6\(lpr\) mice created OT-1\(lpr\) mice. Offspring were screened for the \(lpr\) mutation by PCR, as previously described (18), and for the clonotype TCR using anti-V\(\alpha\)2 mAb.
Abs, cell preparations, and flow cytometry

Monoclonal anti-murine CD8α conjugated to Red613 was purchased from Life Technologies (Gaithersburg, MD). Monoclonal anti-murine CD44 conjugated to Tricolor or PE was purchased from Caltag Laboratories (Burlingame, CA). Monoclonal anti-murine Vα2 conjugated to PE, monoclonal anti-murine CD69 conjugated to PE, monoclonal anti-murine CD80 (B7.1) conjugated to FITC, monoclonal anti-murine CD86 (B7.2) conjugated to PE, and monoclonal anti-murine H-2Kk conjugated to biotin were purchased from BD Pharmingen (San Diego, CA).

Single cell suspensions were made by homogenizing tissues in RPMI 1640 medium (Life Technologies) supplemented with 5% bovine serum (BCS; HyClone Laboratories, Logan, UT). For flow cytometry, 750,000 cells were incubated in 0.1 ml PBS containing 0.5% BSA Fraction V, 0.01% (v/v) sodium azide (PBS-azide) (Sigma, St. Louis, MO). The Abs listed above (3 μg/ml) at 4°C for 30 min. After washing with PBS-azide, cells were fixed in 1% methanol-free formaldehyde (Ted Pella, Reading, CA) in PBS-azide. Samples were stored at 4°C until they were analyzed with a Coulter Elite flow cytometer calibrated using DNA check beads (Coulter, Hialeah, FL).

OVA and treatment of TCR transgenic mice

Peptide to chicken OVA 257–264 (SINNFELK) (OVApeptide) was produced at Macromolecular Resources (Colorado State University, Fort Collins, CO). Mice received two i.p. injections 24 h apart of 100 μl 100 μM peptide solution in PBS or PBS alone. Tissues were harvested 2, 3, 5, or 7 days after the first injection of peptide.

Isolation of liver lymphocytes

Mice were euthanized, and the peritoneal cavity was opened and the portal vein identified. This was cannulated with a 27-gauge needle and perfused with 5–10 ml PBS until all the lobes of the liver blanched. With the needle remaining in the portal vein, the inferior vena cava was cut above the liver. The liver was then excised with forceps, and the gall bladder was identified and removed. The liver was washed once in RPMI/5% BCS and then cut into small pieces and homogenized in a tissue grinder. Cells were then spun at 37°C for 40 min, mixing the tissue frequently. A total of 30 ml serum-free RPMI was then added and spun at 300 rpm for 3 min. This sedimented the majority of hepatocytes, but left lymphocytes in the supernatant. The supernatant was transferred to another 50-ml tube and spun at 1200 rpm for 10 min. The supernatant was aspirated and the cells were then resuspended in a total volume of 1.6 ml serum-free RPMI and transfused to a 15-ml tube containing 1 ml of 2-mercaptoethanol (w/v) mercuric (mercapto) (Sigma) in RPMI was added to the cells and mixed well. This solution was overlaid with 1 ml serum-free RPMI and spun at 2500 rpm for 20 min. Liver lymphocytes were identified at the interface, carefully aspirated, and transferred to another 15-ml tube, then washed with RPMI/5% BCS and spun at 1600 rpm for 10 min. Cells were then resuspended in RPMI/5% BCS for analysis or placed in RPMI containing 25 mM HEPES, 5% FCS, 100 U/ml penicillin, and 100 U/ml streptomycin.

Detection of apoptosis by TUNEL

To analyze apoptotic cells by flow cytometry, the TUNEL assay was used as described (19, 20). Lymphocyte populations were initially incubated in complete medium at 37°C for 4 h. Cells were initially stained for expression of Fas-PE, Cy5, and then fixed for 15 min in 1% formalde- hyde. Cell membranes were then permeabilized for 15 min using 70% ethanol at 4°C. Samples were incubated at 37°C for 1 h in 50 μl containing 10 U TdT and 0.5 nM dUTP–biotin (Roche Diagnostics, Indianapolis, IN). Specimens were washed twice with PBS/1% BSA and incubated with a 1:50 dilution of streptavidin Tricolor (Caltag Laboratories) at 4°C for 30 min. Cells were washed twice and analyzed by flow cytometry. Negative controls were instead staining of cells with the same protocol, but in the absence of dUTP–biotin.

A modification of Gavrieli et al. (21) was used to measure apoptosis in situ. Tissue sections were fixed in 10% neutral buffered Formalin for 24 h and embedded in paraffin, and 5-μm sections were cut and mounted on slides and heated at 60°C overnight. Slides were hydrated by washing twice in 100% ethanol for 5 min each, then 96%, 90%, and 80% ethanol, and finally twice in double-distilled water (ddH2O). Nuclear proteins were stripped by incubating with 25 μg/ml protease K (Sigma) in 10 mM Tris, 5 mM CaCl2, pH 7.4, at room temperature for 15 min, followed by washing in ddH2O. Endogenous peroxidase was inactivated by submerging the slides in 0.6% H2O2 at room temperature for 15 min. Sections were rinsed twice in ddH2O and treated with 30 mM Tris-HCl, pH 7.2, 140 mM sodium cacodylate, 4 mM MgCl2, 0.1 mM DTT, after which, positive control slides were treated with 10 μg/ml DNase I (Sigma) at room temperature for 15 min. All slides were washed three times in ddH2O before the addition of TdT buffer (30 mM Tris-HCl, pH 7.2, 140 mM sodium cacodylate, 1 mM CoCl2). Nick end labeling of DNA was performed by incubating slides with 30 U TdT (Roche Diagnostics) in 100 μl TdT buffer/0.05% BSA at 37°C for 60 min. TdT was omitted from negative control slides. The re- action was terminated by submerging slides in TB buffer (300 mM NaCl, 30 mM sodium citrate) at room temperature for 20 min. Sections were rinsed with ddH2O, covered with 2% BSA in TBS (20 mM Tris-HCl, 150 mM NaCl, 2 mM CaCl2, pH 7.4), and incubated with 100 μl streptavidin- peroxidase (Zymed Laboratories, San Francisco, CA) at room temperature for 20 min. Following three washes of TBS, 90 μl 1:20 aminohydraly carbazole (Zymed Laboratories) and 0.6% H2O2 were added at room temperature for 20 min. Sections were mounted using GVA mounting solution (Zymed Labo- ratories) and photographed with ASA 100 Kodak Gold 135 mm film.

Determination of serum aspartate transaminase levels

Blood from PBS- or OVAp-treated mice was collected from the orbital sinus and centrifuged using Microtainer separator tubes (BD Bio- sciences, Franklin Lakes, NJ). Serum was collected according to the man- ufacturer’s directions. Semiquantitative transaminase levels were deter- mined by colorometric assay conducted by the chemistry laboratory at Fletcher Allen Medical Center (Burlington, VT).

Semiquantitative PCR

A total of 5 × 106 liver lymphocytes was lysed in Ultraspec RNA reagent (Biotecx Laboratories, Houston, TX), and RNA was prepared according to the manufacturer’s directions. Oligo(dT) priming and reverse transcriptase were used to prepare cDNA from RNA samples. PCR amplifications were performed as follows: 94°C × 1 min, 55°C × 1 min, 72°C × 1 min (35 cycles). Primers used for amplification of perforin (22), FasL (23), TNF-α, and hypoxanthine phosphoribosyltransferase (HPRT) (24) have been de- scribed previously. PCR reactions were separated on 1.5% agarose gels, and autoradiographs were analyzed by densitometry (Quantity One software, Biorad, Hercules, CA). Densitometry was quantitated using a digital camera and α Imager software.

Cytotoxicity assays

Adherent Hepa 1.6 cells (American Type Culture Collection, Manassas, VA) were grown in flat 96-well plates. A total of 5 × 105–5 × 106 cells/well was labeled with 5 μCi/ml Na105CrSO4 (New England Nuclear, Boston, MA) for 1 h at 37°C directly in the wells. Wells were washed five times with 200 μl RPMI + 5% BCS. Liver lymphocytes were serially diluted in a separate 96-well plate, followed by a 2-h incubation at 37°C in the presence of 50 nM or 150 nM (final concentrations) concanamycin A (CMA), similarly diluted DMSO, or media alone. Following the incuba- tion, liver lymphocytes were added to wells containing Hepa 1.6 targets. Additionally, 20 μg/ml Fas-Fc (Alexis, San Diego, CA) was added to wells to block FasL-mediated apoptosis. A total of 50–100 μl supernatant was removed at 6 h and 16–18 h, and radioactivity was determined using a gamma counter. HCl (3 N) was diluted 1/1 into select wells to determine maximal 105Cr release. Percent specific lysis was calculated as follows: (experimental cpm – spontaneous release cpm)/(maximum release cpm – spontaneous release cpm) × 100.

Cell surface expression of FasL

A total of 0.5 × 106 lymph node or liver lymphocytes was stained for cell surface expression of FasL using the Enzymatic Amplification Staining Kit (EAS Kit; Flow-Amp Systems, Cleveland, OH). Cells were washed twice with staining buffer (PBS, pH 7.4, 1% BSA, 1% PBS), then incubated at 4°C for 20 min with 6 pg/ml of either isotype control hamster IgG–biotin or hamster anti-murine FasL–biotin (BD Pharmingen). After two washes with staining buffer, all of the cells were incubated in 1:50 dilution of streptavidinHRP secondary reagent (EAS kit) at 4°C for 20 min. Cells were subsequently washed twice with staining buffer, then once with PBS, pH 7.4. Cells were reacted with a 1:20 dilution of amplifier solution (EAS kit) at room temperature for 20 min, followed by two washes with staining buffer. Cells were then stained with directly conjugated Vα2-FITC (BD Pharmingen) simultaneously with streptavidin–PE (Caltag Laboratories) and incubated at 4°C for 20 min. Following two washes with staining buffer, cells were fixed in methanol-free 1% formaldehyde/PBS and stored at 4°C until analyzed by flow cytometry.
Results

Hepatocyte damage is more pronounced in OT-1 than OT-1\textsuperscript{lpr} mice following in vivo OVAp.

Lymph node and spleen composition and cell numbers were very similar between the OT-1 and OT-1\textsuperscript{lpr} mice used in these studies, as was the proliferative capacity to OVAp (data not shown). Following administration of OVAp, the livers of OT-1 mice typically appeared very pale and friable by day 2, which was never observed in the livers of OT-1\textsuperscript{lpr} mice. Furthermore, one of the three OT-1 mice for each time point died on day 2 or 3 in each of the three experiments. By contrast, no OT-1\textsuperscript{lpr} mice died. Paralleling these

FIGURE 1. Similar kinetics of accumulation and decline of liver lymphocytes in OT-1 and OT-1\textsuperscript{lpr} mice administered OVAp. OT-1 (■) and OT-1\textsuperscript{lpr} mice (□) received two 190-μl doses of either PBS or 100 μM OVAp administered i.p. 24 h apart. On the day indicated following the first injection, lymphocytes were isolated from liver, lymph node, and spleen and assessed for the number of Vα2\textsuperscript{+} CD8\textsuperscript{+} and Vα2\textsuperscript{+} CD4\textsuperscript{−} CD8\textsuperscript{+} cells. Shown are the means (± SD) from three experiments, each analyzing two or three mice per time point.

FIGURE 2. Despite comparable liver infiltration of lymphocytes after OVAp administration, hepatocyte damage is more pronounced in OT-1 mice than OT-1\textsuperscript{lpr} mice. Mice received either PBS (A and D) or OVAp (B, C, E, and F), as described in Fig. 1. Livers from OT-1 (A–C) and OT-1\textsuperscript{lpr} mice (D–F) were taken on day 2 or day 3 after the first injection and stained with H&E. B and E, Show the degree of lymphocyte infiltration in the periporal region, which was similar in both groups of mice. C, Reveals extensive hepatocyte damage in OT-1 livers consisting of initial loss of granularity and membrane clarity (left half, filled arrow), progressing to complete loss of cellular definition (right half, open arrow), which was not observed in OT-1\textsuperscript{lpr} mice (F). Magnification, ×400.
findings, OVAp administration induced extensive infiltration of \( \text{Va}^{+} \text{CD8}^{+} \) lymphocytes into the livers of both OT-1 and OT-1\( \text{lpr} \) mice by day 2 (Fig. 1). Importantly, no difference was observed in either the kinetics of the OVAp response or the number of \( \text{Va}^{+} \text{CD8}^{+} \)-infiltrating lymphocytes in the livers of the two types of mice. In addition, the rates of decline of liver lymphocytes after day 2 were essentially identical (Fig. 2).

A very similar pattern of \( \text{Va}^{+} \text{CD8}^{+} \) expansion and contraction was observed in the lymph nodes of both types of mice, except for an isolated increase in OT-1 mice on day 3 that was not observed at other time points, nor in the spleen cells. These findings were consistent over three experiments and highlight that the kinetics of loss of OT-1 T cells was not different on the \( \text{lpr} \) background. We (25) and others (1, 26) have observed the appearance of CD4\(^{+} \) T cells following activation of CD8\(^{+} \) T cells. Although these cells increased in number following OVAp, they did not accumulate in OT-1\( \text{lpr} \) mice (Fig. 1, lower panels).

Fig. 2 shows H&E staining of liver sections before and after OVAp treatment. Livers from OT-1 (Fig. 2A) or OT-1\( \text{lpr} \) (Fig. 2D) mice that received PBS showed a normal morphology of hepatocytes characterized by distinct membranes and vacuolar appearance of the cytoplasm. There were also few lymphocytes within the sinusoids or periportal regions of the liver. However, by day 2 after OVAp, large numbers of lymphocytes infiltrated the livers of both OT-1 and OT-1\( \text{lpr} \) mice. Infiltrating lymphocytes in OT-1 livers were seen clustered initially in the periportal regions (Fig. 2B), followed shortly by migration into the parenchyma by day 3 (Fig. 2C). A similar degree of periportal infiltrates (Fig. 2E) and parenchymal invasion (Fig. 2F) was visible in OVAp-treated OT-1\( \text{lpr} \) mice. Concomitant with lymphocyte infiltration, hepatocyte damage was discernable in livers from primarily OT-1 mice given OVAp. Damaged hepatocytes stained more darkly and displayed a hazy ground-glass morphology with few intracellular vacuoles and indistinct cellular membranes (Fig. 2C, left half). Additionally, areas of more extensive liver damage were frequently observed by pink staining of the cytoplasm with nearly complete loss of cellular detail (Fig. 2C, right half). Markedly less damage was noted in livers from OT-1\( \text{lpr} \) mice given OVAp. In this case, hepatocytes clearly displayed vacuolar cytoplasm and distinct membranes, despite similar degrees of lymphocytic infiltration (Fig. 2E).

Differences in hepatocyte damage were even more evident when liver sections were stained for nicked DNA using the TUNEL method. Three and five days after the initial OVAp injection, extensive hepatocyte death was visible in OT-1 mice (Fig. 3A–C). However, livers from OT-1\( \text{lpr} \) mice given OVAp displayed only minimal damage (Fig. 3D–F). Serum levels of the hepatocyte enzyme aspartate transaminase (AST) also confirmed the differences in liver injury. Mice treated with PBS manifested normal basal AST values (88–200 U/L). Following OVAp administration, however, serum AST values rose dramatically at day 2 in OT-1 mice, but only slightly in OT-1\( \text{lpr} \) mice (Fig. 4). The AST values for OT-1 mice at day 2 were actually an underestimate, as the serum tested gave a reading beyond the 750 U/L upper limit of the linear range of the assay. Despite this, the difference in AST values between the OT-1 and OT-1\( \text{lpr} \) mice on day 2 was highly significant (\( p < 0.0007 \), paired Student’s t test). Additionally, whereas the difference in AST values on day 2 from OT-1 mice given OVAp vs PBS was significant (\( p < 0.0006 \)), the same comparison in OT-1\( \text{lpr} \) mice did not reach statistical significance.

The lack of hepatocyte damage in OT-1\( \text{lpr} \) mice given OVAp, despite similar liver lymphocyte infiltration, is consistent with the fact that these mice lack surface expression of Fas. In this model, CD8\(^{+} \) T cells are activated by OVAp to become effector CTL expressing FasL. A portion of the cells migrates to the liver and induces hepatocyte death. Implicit in this model is the ability of OT-1\( \text{lpr} \) T cells to fully activate in response to OVAp to express cytolytic molecules. This issue was examined further by assessing

![FIGURE 3](image)

**FIGURE 3.** Infiltrating lymphocytes induce extensive hepatocyte apoptosis in OT-1 mice compared with OT-1\( \text{lpr} \) mice. Mice received either PBS or OVAp, as described in Fig. 1. Livers from OT-1 (A–C) and OT-1\( \text{lpr} \) mice (D–F) were analyzed at the day indicated after the first injection and stained for nicked DNA using the TUNEL method. Lower panels are negative controls (no TdT) (G) and positive controls (DNase treated) (H). Magnification, \( \times400 \).

![FIGURE 4](image)

**FIGURE 4.** Elevation of serum AST after OVAp administration is more pronounced in OT-1 mice than in OT-1\( \text{lpr} \) mice. OT-1 and OT-1\( \text{lpr} \) mice received either PBS or OVAp, as described in Fig. 1. Serum AST levels were determined on the day indicated after the first injection. The day 2 AST values for OT-1 mice receiving OVAp were an underestimate, due to the upper limits of the assay, and is marked with a \( > \) symbol. Significant differences were found between OVAp-treated OT-1 and OT-1\( \text{lpr} \) mice analyzed at day 2 (\( p < 0.0007 \), paired Student’s t test), and between OVAp- and PBS-treated OT-1 mice analyzed at day 2 (\( p < 0.0006 \)).
FIGURE 5. Appearance of TUNEL+ and B220+ T cells in the liver. On days 3 and 7 following the first PBS or OVAp injection, lymphocytes from spleen and liver were isolated from OT-1 and OT-1/lpr mice. The Vα2+ subset was analyzed for surface expression of CD4 and CD8 by flow cytometry. Cells were also simultaneously assessed for death by TUNEL assay and expression of B220. Numbers in quadrants represent the percentage of Vα2+ CD8+ cells that are either B220+ or TUNEL+. Shown are the findings from OT-1 mice in one of three experiments. Summary of OT-1 and OT-1/lpr results is shown in Table I.

Liver lymphocytes manifest similar phenotypes and rates of cell death in OT-1 and OT-1/lpr mice

Activated T cells undergo apoptosis by a process known as activation-induced cell death. A phenotypic change that often occurs concomitant with the entry of T cells in apoptosis is the surface expression of B220 (1). As lymphocytes entering the liver have been reported to die (27), differences in hepatocyte damage might merely reflect variability of lymphocyte death and the release of proteases. Thus, the TUNEL assay and B220 expression were used to assess the kinetics and degree of lymphocyte death following OVAp.

CD8+ liver lymphocytes for control PBS-treated mice contained high proportions of TUNEL+ as well as B220+ cells compared with splenocytes from the same mice (Fig. 5 and Table I). There was little difference between OT-1 and OT-1/lpr mice for these parameters (Table I). This is consistent with the view that most of the lymphocytes entering the liver are T cells. However, the initial wave of CD8+ cells entering the liver on days 2 and 3 after OVAp administration manifested few TUNEL+ or B220+ cells, whereas the CD8+ cells in spleens at the same time showed marked expansions of both TUNEL+ and B220+ cells (Fig. 5). Thus, the initial wave of hepatic lymphocytes was not dying in either group of mice. However, by day 7, as liver lymphocyte numbers diminished, there was a return to initial levels of both TUNEL+ and B220+ cells (Table I). Throughout this 7-day period following OVAp administration, there was little difference between OT-1 and OT-1/lpr liver lymphocytes in either the kinetics or magnitude of TUNEL+ and B220+ cells (Table I). These findings are in agreement with the similar kinetics of liver lymphocyte infiltration and decline between OT-1 and OT-1/lpr mice. Furthermore, following OVAp in OT-1/lpr mice, there was no sustained accumulation of B220+ cells in either lymph node, spleen, or liver (Table I, and data not shown). This raises questions regarding to what extent the accumulating B220+ cells in lpr mice result from an Ag-driven process.

Decreased OT-1/lpr hepatocyte death is not due to diminished cytolytic capacity of OT-1/lpr liver lymphocytes

Cytotoxic T cells kill targets primarily through a process dependent on perforin, TNF-α, or FasL (28). It was thus possible that differences in hepatocyte death between OT-1 and OT-1/lpr mice might result from differences in their ability to produce these effector molecules. This was examined in two ways, either by measuring direct cytolytic potential of liver lymphocytes in vitro, or by their expression of mRNA for perforin, TNF-α, and FasL. The hepatoma cell line Hepa 1.6 (H-2b) was used as a syngeneic target to determine which cytolytic pathways were activated in liver lymphocytes from mice given OVAp. Freshly isolated liver lymphocytes from day 2 OVAp-treated OT-1 or OT-1/lpr mice killed Hepa 1.6 targets even in the absence of exogenously added OVAp (Fig. 6A). This contrasted with minimal cytolytic activity by spleen or lymph node T cells from the same mice (data not shown). In addition, there was very little cytolyis by liver lymphocytes from PBS control mice (Fig. 6A). Liver lymphocytes from OT-1/lpr mice were consistently slightly more cytolytic than those from OT-1 mice in three separate experiments. The lack of a requirement of the functional state of CD8+ cells in the liver as well as their expression of the cytolytic effector molecules TNF-α, perforin, and FasL.

Table I. Appearance of dying TUNEL+ B220+ cells lymphocytes after OVAp administration

<table>
<thead>
<tr>
<th>Table I. Appearance of dying TUNEL+ B220+ cells lymphocytes after OVAp administration</th>
<th>OT-1 Va2+CD8+</th>
<th>OT-1/lpr Va2+CD8+</th>
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<tr>
<td></td>
<td>Spleen</td>
<td>Liver</td>
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<tr>
<td>Condition</td>
<td>% B20+</td>
<td>% TUNEL+</td>
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<tr>
<td>PBS day 3</td>
<td>25</td>
<td>30</td>
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<tr>
<td>OVA day 3</td>
<td>38</td>
<td>48</td>
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<td>OVA day 7</td>
<td>24</td>
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Mice received injections i.p. of either PBS or OVAp and on the days indicated postinjection, splenocytes and liver lymphocytes were isolated and analyzed for the proportion of Va2+CD8+ cells that expressed either surface B220 or nicked DNA by the TUNEL assay. Similar results were observed in two additional experiments.
exogenous OVA Ag for cytolyis of the Hepa 1.6 targets was supported by the ability of liver lymphocytes to also efficiently lyse allogeneic P815 (H-2d) target cells (Fig. 6B). OT-1 T cells do not manifest natural cross-reactivity to H-2b. This suggested that liver lymphocytes expressed lytic molecules when freshly isolated and did not require further Ag stimulation.

Blocking studies using the perforin inhibitor, CMA (29, 30), partially blocked killing of Hepa 1.6 cells equivalently by both OT-1 mice and OT-1lpr liver T cells (Fig. 6C). Fas-Fc also partially blocked target killing to similar degrees in both mice (Fig. 6C). This is consistent with the view that both FasL and perforin were partly responsible for the lysis of the hepatocytes. Positive control for Fas-Fc blocking of FasL killing was defined using FasL-expressing 293 T cells (Fig. 6D). To confirm that the perforin pathway was activated in T cells from mice given OVA Ag, further cytolyis assays were performed using the thymoma target EL4 that is not sensitive to FasL-mediated apoptosis (N. J. Kennedy, unpublished observations). Liver lymphocytes isolated from OT-1 or OT-1lpr mice given OVA Ag killed EL4 targets, and this was completely blocked by CMA, but unaffected by Fas-Fc (Fig. 6E).

By contrast, anti-TNF-α did not block cytolytic activity from liver lymphocytes of either OT-1 or OT-1lpr mice (data not shown). These observations show that liver lymphocytes from mice administered OVA Ag kill targets in vitro using both the perforin- and FasL-mediated pathways, and support the view that the same pathways are involved with liver damage in vivo in OVA Ag-treated OT-1 mice.

To further assess the cytolytic capacity of liver lymphocytes from OT-1 vs OT-1lpr mice, semiquantitative PCR was performed to assess the levels of expression of perforin, TNF-α, and FasL, using the levels of expression of the hprt gene as a comparison. An example of the PCR results of cDNA titrations from liver lymphocytes is illustrated in Fig. 7A and summarized in Fig. 7B for all three experiments analyzed. A striking finding was the significantly higher levels of message for perforin, TNF-α, and FasL, and in liver lymphocytes compared with lymph node or spleen cells from the same animals. As T cells in lymph nodes, spleens, and livers in OT-1 mice are nearly all CD8+; these differences did not reflect different composition of CD4+ vs CD8+ cells. This underscores the notion that liver CD8+ lymphocytes are enriched for activated T cells following Ag stimulation. A less striking feature was the slightly increased expression in lymph nodes of perforin, TNF-α, and FasL by day 2 after OVA Ag. A somewhat opposite trend was apparent in the spleen. These trends paralleled the initial increase of Va2+ CD8+ T cells in lymph nodes after OVA Ag, but their rapid decline in number in the spleen. Conceivably, the activated splenic T cells may have rapidly migrated to the liver. There were only minimal nonstatistical differences between both strains of mice in message levels of these molecules within liver lymphocytes. This further supports the view that the difference in hepatocyte cell death in vivo was not the result of diminished cytolytic capacity of OT-1lpr CD8 cells. Finally, in lymph node and spleen cells, there was a moderate increase in message for

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**FIGURE 6.** Lymphocytes that infiltrate the liver following OVA Ag administration possess direct cytolytic activity when freshly isolated. Mice received injections of PBS or OVA Ag, as described in Fig. 1, and liver lymphocytes isolated on day 2 postinjection. A, Liver lymphocytes from OT-1 mice (squares) or OT-1lpr mice (circles) that received either OVA Ag (closed symbols) or PBS (open symbols) were assayed for cytolytic activity in a 51Cr release assay using syngeneic (H-2b) Hepa 1.6 target cells in the absence of OVA Ag. Comparison is shown to lysis by 3T3 fibroblasts transfected with FasL (●). No cytolytic activity was observed in lymph node or spleen cells from the same mice (data not shown). B, Cytolyis of allogeneic P815 (H-2d) target cells by liver lymphocytes. C, Inhibition of cytolytic activity by Fas-Fc (20 μg/ml) to block FasL, or by CMA (150 μM) to block perforin. Cytolyis is expressed as the percentage of maximal killing observed by lymphocytes in the presence of control Ig (20 μg/ml). D, Lysis of Hepa 1.6 cells by FasL-transfected 3T3 cells in the presence of control Ig (20 μg/ml), Fas-Fc (20 μg/ml), or CMA (150 μM). E, Lysis of Fas-resistant EL-4 cells by liver lymphocytes in the presence of control Ig, Fas-Fc, or CMA.
FasL from OT-1/lpr mice compared with OT-1 mice. This is in agreement with earlier findings (31) and might reflect a compensation by lymphocytes for the lack of Fas (32, 33).

Consistent with the mRNA studies for FasL, surface FasL was considerably induced on liver lymphocytes by day 2 after OVAp (Fig. 7C). These levels were also higher than from lymph nodes from the same day 2 animals, especially OT-1 mice. Surface FasL was also much higher on OT-1/lpr lymph node T cells than from OT-1 mice.

### Discussion

The current findings extend our earlier results that Ag-activated CD4" and CD8" T cells migrate to the liver, but that liver damage is preferentially caused by CD8" cells (5). OVAp-activated OT-1 CD8" liver lymphocytes used the perforin and Fas mechanisms of cell death in vitro to kill target hepatocytes. This was comparable between OT-1 and OT-1/lpr liver lymphocytes as defined by both functional assays and PCR analysis of effector molecules. There was a moderately increased expression of FasL by lpr T cells, which may explain the slightly more efficient killing of the Hepa 1.6 target cells by OT-1/lpr liver lymphocytes in vitro. Nonetheless, considerably less hepatocyte damage was observed in vivo in OT-1/lpr mice compared with OT-1 mice following OVAp. These findings underscore the importance of the Fas death pathway in this model of liver injury.

Although hepatocytes are clearly sensitive to death following in vivo injection of anti-Fas Ab (10), it has been less certain to what

![FIGURE 7. Liver lymphocytes display high basal mRNA levels of TNF-α, perforin, and FasL. OT-1 and OT-1/lpr mice received either PBS or OVAp, and liver lymphocytes were isolated at the times indicated after OVAp and cDNA prepared. Semi-quantitative PCR was normalized to levels of hprt message. A, Representative PCR for the indicated genes using serial 2-fold dilutions of cDNA. B, Relative levels of gene expression normalized to hprt expression. Densitometry was performed on PCR products from serial dilutions of cDNA, as shown in A. The dilution of cDNA yielding 50% of maximal hprt density was used as the standard against which to normalize the 50% maximal density of the indicated gene product. C, Surface FasL expression detected by Flow Amp system of lymph nodes and liver lymphocytes from the same mice that received either PBS or OVAp 2 days previously. Inserts show control staining on Vα2" cells for FasL using hamster Ig](http://www.jimmunol.org/Downloadedfrom)
extent Fas contributes to hepatocyte damage by liver-infiltrating lymphocytes. In three studies using in vivo Con A, liver damage induced by T cells was mediated by perforin or IFN-γ, but not by FasL (11, 12, 34). Other studies using viral-specific T cells from mice bearing transgenically expressed hepatitis B virus yielded conflicting results, either supporting a role for Fas in liver damage (15), or arguing against any involvement of Fas (14, 35). These differences may have resulted in part from the heterogeneity of infiltrating T cells. However, in none of these studies were liver lymphocytes isolated and studied directly for their cytolytic activity or expression of cytolytic effector molecules. The current studies represent the first analysis of the cytolytic activity and mechanisms used by the actual infiltrating lymphocytes in the liver. Hepatocyte damage in OT-1 mice given OVAp results from both perforin- and FasL-mediated killing pathways, as both the perforin inhibitor CMA as well as Fas-Fc block in vitro killing of hepatocyte targets by OT-1 liver lymphocytes. The partial involvement of perforin would be consistent with our findings in vivo that OT-1lp/lpr mice were not completely devoid of histological evidence of damage of hepatocytes or elevation of serum AST, although it was dramatically less than in Fas-bearing OT-1 mice.

Liver lymphocytes were directly cytolytic when freshly isolated and did not require in vitro restimulation with OVAp, unlike spleen or lymph node T cells from the same mice. This is consistent with the view that the CD8+ cells may become activated in peripheral lymphoid tissues, but then migrate to the liver, where cytolytic activity would become concentrated. This model would suggest that OVAp-induced liver injury in mice represents an “innocent bystander” phenomenon. In this system, Ag presentation by the target organ may not be necessary for either the trafficking of lymphocytes to the organ or the subsequent tissue injury induced by infiltrating lymphocytes. We (J. Q. Russell, unpublished observations) and others (2) have observed that Ag-activated T cells also traffic to the lung and kidneys, and we are examining the degree of tissue injury that results at these sites. This model might also serve to explain the liver dysfunction that is often observed after situations in which the immune system has been strongly activated largely outside the liver, such as by a superantigen in toxic shock syndrome (36, 37). It also suggests that the normal number of resident liver lymphocytes in wild-type mice might induce tonic low levels of hepatocyte apoptosis in a Fas-dependent manner. This would be consistent with the observation that Fas-deficient mice have larger livers than wild-type mice (38).

The current findings also raise questions regarding the origin of the CD4+ CD8- B220- T cells that accumulate in lpr mice. Prevailing evidence suggests that these unusual lpr T cells derive from a CD8+ precursor. This is supported by persistent demethylation of the CD8α gene in lpr CD4+ CD8- T cells (39), and the absence of these cells in lpr mice deficient in β2-microglobulin (18, 40). Although we have previously observed that administration of OVAp to OT-1 mice results in the transient appearance of Vα2+ CD4+ CD8- T cells (25), the current studies did not observe an accumulation of these cells in OT-1lp/lpr mice. Conceivably more persistent administration of OVAp to OT-1lp/lpr mice might result in accumulation of Vα2+ CD4+ CD8- T cells. However, even with the limited administration of OVAp in this protocol, Vα2+ cells, either CD8+ or CD4+CD8-, were largely eliminated from lymphoid tissues of OT-1lp/lpr mice by day 7 with kinetics that was nearly identical to OT-1 mice. These findings suggest that the derivation of CD4+ CD8- T cells from CD8+ precursors in lpr mice may not result from chronic antigenic activation, but by a different process.

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References


