Hepatocytes Can Serve as Accessory Cells in the Response of Immune T Lymphocytes to Heat-Killed *Listeria monocytogenes*

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Received 12 October 1994/Returned for modification 21 November 1994/Accepted 12 December 1994

Previous findings in our laboratory indicated that the bulk of *Listeria monocytogenes* injected intravenously into mice and recovered in the liver is taken up and replicates within hepatocytes. Other investigators have shown that hepatocytes can display costimulatory adhesion molecules, express major histocompatibility complex class I and II molecules, and secrete a number of cytokines, including interleukin-1 (IL-1), IL-6, and IL-8. These data suggest that hepatocytes may serve as accessory cells in the immune response to *L. monocytogenes*. The accessory function and capacity of hepatocytes to present listerial antigens, however, have never been explored. We undertook a series of experiments to examine the response of *Listeria*-immune T lymphocytes to murine hepatocytes preincubated with heat-killed listeriae (HKL). Electron micrographs showing the organism within membrane-limiting vacuoles demonstrated the capacity of hepatocytes to internalize HKL. T cells cocultured with hepatocytes pulsed with HKL exhibited a 5- to 10-fold increase in [*methyl*-³H]thymidine incorporation relative to T cells cultured with either hepatocytes or HKL alone. Similarly, gamma interferon production by immune T cells was elevated significantly in cultures that contained both hepatocytes and HKL. The optimal response of T cells required lysosomal processing of HKL by hepatocytes and contact between the two cell populations. Furthermore, maximum T-cell proliferation and gamma interferon production were dependent upon the presence of CD4⁺ T lymphocytes and the expression of Ia antigens. Taken together, these findings demonstrate that hepatocytes pulsed with HKL can stimulate the antigen-specific response of immune T lymphocytes. These results suggest that hepatocytes can serve as accessory cells in host defenses to listerial infections of the liver.

Most of the bacteria that enter the bloodstream are taken up by the liver (5, 29). Although alterations in the histology and function of the liver occur frequently as a consequence of sepsis, bacteria are rarely cultured from biopsies (7). The mechanisms underlying clearance of the blood and sterilization of the liver remain to be determined.

Listeriosis in mice is an experimental model used widely to study the mechanisms that underlie host defenses to intracellular pathogens (20). Recently we reported that the bulk of *Listeria monocytogenes* injected intravenously into an animal and taken up in the liver was associated with the hepatocyte population at 2 h postinfection (15). Hepatocytes are the principal site of listerial replication in the liver (15, 32). Nonimmune mice infected with a sublethal dose exhibited a 200-fold increase in the number of listeriae within the hepatocytes by 3 days postinfection (15). Listeriae injected intravenously into immune animals, on the other hand, were taken up by hepatocytes and rapidly eliminated.

Recovery from primary listerial infections and protective immunity to subsequent infections are mediated, in part, by antigen-specific T lymphocytes (20, 22, 31). Both CD4⁺ (helper, major histocompatibility complex [MHC] class II-restricted) and CD8⁺ (cytotoxic/suppressor, MHC class I-restricted) T-cell subpopulations have been implicated (20–22). CD8⁺ T cells, however, play the predominant role. Using

monoclonal antibodies to eliminate specific immune T-cell subsets, investigators showed that mice adoptively immunized with CD4-depleted (i.e., CD8-enriched) T splenocytes exhibited the same degree of resistance as did mice immunized with the total splenocyte population (3, 10). Antigen-specific CD4⁺ T cells are also capable of transferring resistance to listeriae, albeit much less efficiently (3, 21, 27).

Hepatocytes can exhibit class I and II MHC molecules (12, 13), display costimulatory adhesion molecules (1, 28, 39, 40), express receptors for cytokines such as gamma interferon (IFN- γ), interleukin-6 (IL-6), and tumor necrosis factor alpha (14, 34, 42), and secrete a variety of soluble immunologically active factors, including IL-1, IL-6, IL-8, macrophage and granulocyte-macrophage colony-stimulating factors, and acute-phase proteins (33–36). These findings have led others to suggest that hepatocytes might serve as immunomodulating (accessory) cells capable of initiating and perpetuating immune responses occurring within the liver (41). The accessory function of hepatocytes remains to be delineated. Here we report that murine hepatocytes preincubated with heat-killed listeriae (HKL) stimulated the antigen-specific proliferation and production of IFN- γ by immune, CD4⁺ T lymphocytes. The response of T lymphocytes was MHC II restricted and dependent upon lysosomal function and required direct contact between the two cell populations.

MATERIALS AND METHODS

Bacteria. *L. monocytogenes* EGD was cultured and maintained as described previously (43). The bacterium was passed periodically in mice to sustain its virulence. Bacteria were bulk cultured in trypticase soy broth, harvested, washed,

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and stored at -70°C as stock. HKL were prepared by incubating the bacteria in a 60°C water bath for 1 h and were stored at -70°C for use. *Salmonella typhimurium* C₅S (obtained originally from Frank Collins, Trudeau Institute, Saranac Lake, N.Y.) was maintained as described previously (43).

Mice. Eight- to 12-week-old, specific-pathogen-free female C57BL/6J mice (H-2^b; Jackson Laboratories, Bar Harbor, Maine) were the source of all cells described in this report. Mice were maintained in barrier cages with filtered inflow air. Mice were immunized by intravenous injection of 10^4 listeriae 10 to 13 days before use.

Preparation and characterization of hepatic cells. Hepatocytes and nonparenchymal cells (NPC) were prepared following perfusion of mouse livers, using a two-step technique described elsewhere (15, 17). Briefly, the livers of anesthetized animals were perfused with warm (42°C) Hanks' balanced salt solution (free of Ca^{2+} and Mg^{2+}) followed by warm collagenase (100 U/ml, type B; Boehringer Mannheim, Indianapolis, Ind.) in Leibovitz's modified L-15 medium (GIBCO Laboratories, Grand Island, N.Y.). The perfused livers were dissected, and the gallbladders were removed. The livers were teased apart in L-15 medium with forceps, and the suspension was passed through several layers of gauze. The resultant cell suspension was centrifuged at $30 \times g$ for 4 min at 4°C . The parenchymal cells recovered in the pellet were washed once by low-speed centrifugation. The purified population was judged to be $\geq 96\%$ hepatocytes on the basis of morphology and 98% viable by trypan blue exclusion.

The NPC which remained in suspension following the first low-speed centrifugation of the total hepatic cell population were purified by centrifugation on a 16% metrizamide gradient as described previously (16). The purified NPC population consisted of approximately 30% Mac-1⁺ Kupffer cells, 20% Mac-1⁺ monocytes, lymphocytes, and endothelial cells.

The presence of contaminating dendritic cells and mononuclear phagocytes in the purified hepatocyte population was assessed by immunohistochemistry. Cytochrome preparations of freshly isolated hepatocytes were air dried at room temperature and fixed with methanol at -20°C . Fixed preparations were pre-treated with 0.5% H_2O_2 to inactivate endogenous peroxidase, solutions of avidin and biotin (Vector Laboratories, Inc., Burlingame, Calif.) to block intrinsic biotin, and 10% normal goat serum to limit the nonspecific binding of antibody. The slides were incubated with monoclonal rat immunoglobulin G2b (IgG2b) anti-mouse macrophage (F4/80 hybridoma; American Type Culture Collection [ATCC], Rockville, Md.) or monoclonal hamster IgG anti-mouse CD11c (N418 hybridoma; ATCC), which reacts strongly with dendritic cells in mouse liver (44). Washed slides were then incubated sequentially with biotin-conjugated goat anti-rat (or goat anti-hamster IgG), avidin-biotinylated peroxidase complex, and diaminobenzidine- H_2O_2 (all components obtained from Vector Laboratories). The slides were counterstained with hematoxylin. Microscopic examination of cytopins derived from three different purified hepatocyte populations revealed 1.5% F4/80-positive (macrophages) cells and 0% N418-positive (dendritic) cell among 1,000 cells counted per preparation. In the latter case, a dendritic cell-enriched population obtained from nonparenchymal mouse liver cells by the method of Woo et al. (45) contained 45% N418-positive cells when stained in an identical fashion.

As an alternative method, mononuclear phagocyte contamination of purified hepatocyte preparations was assessed on the basis of the capacity of phagocytes to internalize latex beads (16). The hepatocytes were mixed with a 0.2% suspension of latex beads (1.1- μm diameter; Sigma Chemical Co., Inc., St. Louis, Mo.), centrifuged at $400 \times g$ for 10 min, and incubated for 30 min at 37°C . The cells were resuspended and washed twice by centrifugation through calf serum to remove unincorporated beads. Washed cells were sedimented onto glass slides with a cytospin centrifuge, stained with Wright-Giemsa stain, and examined by phase-contrast microscopy. The percentage of phagocytes was calculated from the number of cells which contained latex beads in a population of 200 cells counted. In three different preparations, phagocytes constituted $\leq 2\%$ of total cells in the purified hepatocyte population.

Electron microscopy. Mouse hepatocytes (3×10^6) were seeded into a 10-cm-diameter tissue culture dish, and the cells were cultured for 2 h. The plate was subsequently inoculated with 3×10^8 HKL and reincubated for 4 h. The cell monolayer was washed four times with Hanks' balanced salt solution, and the cells were fixed in situ with 2.0% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4). The fixed cells were scraped into suspension, postfixed with 1% osmium tetroxide, and embedded in epoxy resin. Thin sections were cut, stained with lead citrate and uranyl acetate, and examined by electron microscopy (Philips model 201 electron microscope).

Preparation and phenotyping of splenic cell populations. Splenic cells were prepared as follows. Dissected spleens were perfused with RPMI 1640 and disrupted with a syringe plunger. The single-cell suspension was collected, the tissue debris was removed, and the erythrocytes were lysed by NH_4Cl treatment (6). The resultant splenocytes were washed twice by centrifugation and then passed twice over nylon wool columns to eliminate macrophages and B lymphocytes (19). The resultant splenocytes are defined as total T cells.

CD4- and CD8-enriched T-lymphocyte populations were obtained by two cycles of complement depletion (26). Nylon wool-enriched T cells were incubated with 50 μg of monoclonal rat anti-mouse CD4 or rat anti-mouse CD8 per ml on ice and then incubated with a 1:20 dilution of LOW-TOX-M rabbit complement (Cedarlane Laboratories Limited, Ontario, Canada) for 30 min at 37°C .

The compositions of the total, CD4-enriched, and CD8-enriched T-cell populations were determined by fluorescence-activated cell sorting (FACS) analysis on a Becton Dickinson Flow Cytometer (Becton Dickinson Immunocytometry Systems, Mountain View, Calif.). In all cases, the cells were suspended in FACS medium (0.1% bovine serum albumin and 0.1% sodium azide in Hanks' balanced salt solution), incubated on ice for 30 min, and washed twice with FACS medium. For detection of total T cells in the nylon wool-enriched population, 10^6 cells were stained with 1 μg of fluorescein isothiocyanate (FITC)-conjugated rat anti-mouse Thy 1.2 monoclonal antibody. To identify CD4⁺ or CD8⁺ T lymphocytes, the cells were incubated with rat anti-mouse CD4 or rat anti-mouse CD8 at 50 $\mu\text{g}/\text{ml}$ and then with 1:50 dilution of FITC-conjugated mouse anti-rat IgG. FITC-conjugated goat anti-mouse IgG/A/M at 1:100 was used to detect B cells. To enumerate natural killer (NK) cells, the cells were first incubated with 5% normal mouse serum in FACS medium and then stained with biotin-conjugated mouse anti-mouse NK 1.1 monoclonal antibody at 1:30 followed by streptavidin-FITC at 1:100. The results of FACS analyses showed that the total immune T-cell population was composed of 87% Thy 1.2⁺ T cells, 4% B cells, and 4.5% NK cells. The CD4-enriched T-cell preparations were 76.2% CD4⁺ and 3.6% CD8⁺. The CD8-enriched T-cell population was 71.4% CD8⁺ and 4.2% CD4⁺.

Monoclonal antibodies and staining reagents were purchased from the following sources: FITC-conjugated rat anti-mouse Thy 1.2, biotin-conjugated mouse anti-mouse NK 1.1, and streptavidin-FITC from Pharmingen, San Diego, Calif.; FITC-conjugated goat anti-mouse IgG/A/M from Cappel, West Chester, Pa.; and FITC-conjugated mouse anti-rat from Jackson ImmunoResearch Laboratories Inc., West Grove, Pa. Ascites fluid from pristane-primed, homozygous nude BALB/c mice injected with 10^7 GK1.5 or 2.43 hybridoma cells (ATCC) was used as the source of monoclonal rat IgG2b anti-mouse CD4 and rat IgG2b anti-mouse CD8, respectively. The antibodies were purified by affinity chromatography on a protein G column (Boehringer Mannheim) according to the supplier's instruction.

Hepatocytes and T-cell cocultures. Hepatocytes suspended in L-15 medium containing 1 mg of glucose per ml and 10% heat-inactivated fetal bovine serum (HyClone Laboratories, Logan, Utah) were gamma irradiated (3,000 rads from a ^{137}Cs source) to prevent cell proliferation. Hepatocytes were seeded into 96-well flat-bottom microtiter plate and incubated for 2 h at 37°C in a humidified environment composed of 5% CO_2 in air. HKL in 0.1 ml of RPMI 1640 with 10% fetal bovine serum was then added to selected wells. The plates were centrifuged at $250 \times g$ for 5 min and incubated at 37°C for an additional 4 h. At the end of the incubation period, the plates were washed gently three times with 0.15 ml of RPMI 1640 to remove the extracellular bacteria.

T cells were suspended in RPMI 1640 medium supplemented with 10% fetal bovine serum, 1 mM sodium pyruvate, 1 mM L-glutamine, 5×10^{-5} M 2-mercaptoethanol, and 5 μg of gentamicin per ml (cloning medium). T cells (2×10^5) were then transferred to wells with or without hepatocytes. The cells were incubated for 4 days at 37°C ; 1 μCi of methyl- ^3H thymidine (^3H TdR; 6.7 Ci/mmol; ICN Radiochemicals, Irvine, Calif.) per well was added for the last 18 h. At the end of the incubation period, aliquots of the culture supernatants were collected for IFN- γ measurement. The cells were then harvested onto glass fiber filters by using a PHD Cell Harvester (Cambridge Technology, Inc., Watertown, Mass.), and the amount of radioactivity incorporated was assessed by liquid scintillation counting (Packard Instrument Co., Downer Grove, Ill.). Results are the means $\pm$ standard deviations (SD) of six replicate cultures.

To determine whether direct cell-cell contact was required in order for immune T cells to respond to antigen-pulsed hepatocytes, Anocell 10 tissue culture plate inserts (Whatman Inc., Clifton, N.J.) were used to separate the two cell types. Hepatocytes (6×10^4) in L-15 medium were added to wells of 24-well plates for 2 h and then pulsed with HKL for 4 h. After the cell monolayer was washed as described above, 6×10^5 immune T cells were added to inserts placed in the wells, and the cells in 0.6 ml of cloning medium were incubated for 4 days; each well was pulsed with 3 μCi of ^3H TdR for the last 18 h. At the end of the incubation period, sample supernatants were collected for IFN- γ determination. The cells in wells and inserts were resuspended, combined, and harvested onto glass fiber filters. The radioactivity incorporated was determined by liquid scintillation counting.

IFN- γ detection by ELISA. The concentration of IFN- γ in culture supernatants was determined by enzyme-linked immunosorbent assay (ELISA). The rat IgG1 anti-mouse IFN- γ capture antibody was purified by affinity chromatography from the ascites fluid of pristane-primed, homozygous nude BALB/c mice injected with 10^7 R4-6A2 hybridoma cells (ATCC). The biotinylated rat anti-mouse IFN- γ detection antibody and avidin-conjugated peroxidase were purchased from Pharmingen and Sigma, respectively. The assay was performed according to a protocol provided by Pharmingen. The concentrations of IFN- γ in the culture supernatants were calculated from standard curves generated with each assay.

Statistical analyses. Results were analyzed by using the SigmaStat statistics program (Jandel Scientific, San Rafael, Calif.). Data derived from three or more groups were compared by one-way analysis of variance or Kruskal-Wallis one-way analysis of variance on ranks; significant differences between two groups were determined with a nonpaired Student's *t* test, a Student-Newman-Keuls test, or a Mann-Whitney rank sum test as noted in the text. *P* < 0.05 was considered significant.

FIG. 1. Electron micrograph of hepatocytes with intracellular HKL. A primary mouse hepatocyte culture was inoculated with HKL (100-to-1 ratio of bacteria to cells). Following 4 h incubation, the cells were fixed and processed for electron microscopy. Intracellular HKL is indicated by the arrow. Original magnification, $\times 13,250$.

RESULTS

Hepatocytes internalize HKL. The ability of hepatocytes to bind and internalize soluble compounds (e.g., asialoglycoproteins) as well as particulate matter (e.g., apoptotic liver cells and viable listeriae) is well documented (2, 9, 11, 17). To demonstrate the capacity of hepatocytes to take up HKL, primary cultures of murine hepatocytes were incubated for 4 h with HKL, fixed, and processed for electron microscopy. Bacteria contained within membrane-limiting vacuoles of hepatocytes were evident upon examination of thin sections (Fig. 1).

Antigen-specific response of immune T cells to hepatocytes preincubated with HKL. To determine the capacity of murine hepatocytes to serve as accessory cells, we measured the antigen-specific proliferative response of *Listeria*-immune T lymphocytes to hepatocytes preincubated with HKL. The antigen-specific proliferation of immune T cells was dependent upon the presence of hepatocytes. Maximum incorporation of isotope was observed when 2×10^5 immune T cells were incubated with 1×10^4 to 3×10^4 hepatocytes pulsed with HKL (Fig. 2). Consequently, T cells were routinely cocultured with 2×10^4 hepatocytes per 0.6-mm well in subsequent experiments.

Antigen concentration also influenced the proliferation of immune T lymphocytes cocultured with hepatocytes. Proliferation peaked in wells pulsed with 10^6 HKL (Fig. 3). HKL over a broad range of concentrations had comparatively little effect on the incorporation of [3 H]thymidine by T cells cultured

FIG. 2. Proliferation of T cells to HKL depends on hepatocytes. Hepatocytes were seeded into wells at increasing concentrations and pulsed with 10^6 HKL. Subsequently, 2×10^5 immune T cells were added, and the cells were cocultured for 4 days. Results are the mean $\pm$ SD counts per minute incorporated in six parallel wells obtained in a single experiment representative of five experiments.

FIG. 3. Titration of antigen. Hepatocytes (2×10^4 per well) were preincubated for 4 h with increasing concentrations of HKL; 2×10^5 immune T cells were then added, and the cells were cocultured for 4 days. Results are the mean $\pm$ SD counts per minute of [3 H]TdR incorporated in an experiment representative of two experiments. In all cases, T lymphocytes cocultured with hepatocytes pulsed with HKL (HKL-HC/IT) incorporated significantly more [3 H]TdR than did T cells cultured alone with an equivalent concentration of antigen (HKL/IT) ($P < 0.005$ [Mann-Whitney rank sum test]).

alone. In the experiments that follow, hepatocytes were pulsed with 10^6 HKL prior to the addition of T lymphocytes.

In addition to proliferating, immune T cells cocultured with HKL-pulsed hepatocytes produced an increased amount of IFN- γ . IFN- γ production was significantly higher than that found in cultures of immune T cells incubated with either hepatocytes or HKL alone (Table 1). The antigenic specificity of the response of *Listeria*-immune T cells is demonstrated by the fact that immune T cells cocultured with hepatocytes pulsed with heat-killed salmonellae neither proliferated nor

TABLE 1. Hepatocytes pulsed with HKL stimulate antigen-specific proliferation and the production of IFN- γ by *Listeria*-immune T lymphocytes

Cell culture ^a	[3 H]TdR incorporation (10^3 cpm; mean $\pm$ SD) ^b	IFN- γ concn (U/ml)
Expt 1		
IT + HKL-HC	114.8 $\pm$ 7.8	148.0
IT + HC	13.9 $\pm$ 2.7*	8.0
IT + HKL	11.6 $\pm$ 5.1*	ND ^c
IT + HKS-HC	20.0 $\pm$ 2.7*	12.0
Expt 2		
IT + HKL-HC	63.1 $\pm$ 1.3	602.2
IT + HC	16.3 $\pm$ 4.7**	14.5
NIT + HKL-HC	13.9 $\pm$ 3.2**	ND
NIT + HC	11.7 $\pm$ 1.3**	ND

^a In experiment 1, *Listeria*-immune T (IT) cells were cocultured for 4 days with hepatocytes preincubated in the absence (HC) or the presence of HKL (HKL-HC) or heat-killed salmonellae (HKS-HC). In experiment 2, *Listeria*-immune T or nonimmune T (NIT) lymphocytes were cocultured for 4 days with hepatocytes (HC) or with hepatocytes pulsed with HKL (HKL-HC).

^b *, significantly less than the counts incorporated by immune T cells cocultured with HKL-HC ($P < 0.05$ [Student-Newman-Keuls test]); **, significantly less than the counts incorporated by immune T cells cocultured with HKL-HC ($P < 0.05$ [Student-Newman-Keuls test]).

^c ND, not detectable.

FIG. 4. NPC are negligible in T-cell proliferation. NPC (800 per well) were pulsed with 10^6 HKL and then cocultured for 4 days with 2×10^5 immune T cells. The results, obtained from a single experiment representative of five experiments, are the mean $\pm$ SD counts per minute of [3 H]TdR incorporated in six wells. HKL-NPC/IT and HKL-HC/IT are significantly different ($P < 0.001$ [Student's t test]). Abbreviations are as defined in the legend to Fig. 3.

produced IFN- γ at levels significantly higher than background. Similarly, nonimmune T cells cocultured with HKL-pulsed hepatocytes failed to proliferate or to produce a detectable amount of IFN- γ .

Contaminating NPC do not account for the response of T cells to HKL-pulsed hepatocytes. Hepatocytes composed $>96\%$ of the purified parenchymal liver cell population used in these studies. Dendritic cells and mononuclear phagocytes account for 0% and $\leq 2\%$ of the cells, respectively (Materials and Methods). Experiments were undertaken to exclude the possible role of contaminating NPC, i.e., mononuclear phagocytes, in the T-cell response observed in our coculture system. NPC, equivalent in number to the maximum detected in our purified hepatocyte population (i.e., 800 NPC per well), were seeded into flat-bottom microtiter plates and pulsed with HKL. Immune T lymphocytes cocultured with antigen-pulsed NPC incorporated fivefold less [3 H]TdR than did T cells incubated with hepatocytes precultured with HKL (Fig. 4). Similarly, the supernatants derived from cocultures composed of antigen-pulsed NPC rather than antigen-pulsed hepatocytes contained significantly less IFN- γ (Fig. 5). These findings indicate that contaminating NPC played a negligible role in the increased proliferation and secretion of IFN- γ by *Listeria*-immune T lymphocytes cocultured with hepatocytes and HKL.

NH₄Cl blocked the response of immune T cells to antigen-pulsed hepatocytes. NH₄Cl is a lysosomotropic agent that alters the pH of endosomes and phagolysosomes (46). Treatment with NH₄Cl inhibits the processing and presentation of exogenous, particulate antigens by macrophages. As a consequence, NH₄Cl-treated macrophages exhibit a markedly reduced capacity to initiate the antigen-specific response of T lymphocytes (46). To determine whether lysosomal processing was an obligate requirement for the response of *Listeria*-immune T lymphocytes to hepatocytes pulsed with antigen, hepatocytes were preincubated with HKL in the presence or absence of NH₄Cl. As shown in Table 2, both T-cell proliferation and the production of IFN- γ were diminished markedly in cocultures that contained NH₄Cl during the preincubation pe-

FIG. 5. IFN- γ production by T cells cocultured with NPC. The culture supernatants were collected from the cultures described in the legend to Fig. 4, and the concentrations of IFN- γ were determined.

riod with antigen. Treatment with 2 mM NH_4Cl had no effect on the viability of either T lymphocytes or hepatocytes (data not shown).

The response of T cells to hepatocytes preincubated with HKL requires cell-cell contact. To determine whether the response of immune T cells to HKL-pulsed hepatocytes required direct cell-cell contact, T lymphocytes and hepatocytes in tissue culture wells were incubated together or separated by inserts. Immune T lymphocytes incubated in direct contact with HKL-pulsed hepatocytes had elevated levels of proliferation and IFN- γ production (Table 3). On the other hand, immune T cells separated from hepatocytes by inserts exhibited only a background level of proliferation; IFN- γ was undetectable. Thus, direct contact with HKL-pulsed hepatocytes was required for optimal proliferation and IFN- γ production by *Listeria*-immune T lymphocytes.

The response of immune T lymphocytes to HKL-pulsed hepatocytes requires the expression of MHC class II molecules. The response of immune T lymphocytes to HKL processed and presented by macrophages is generally associated with the expression of class II MHC molecules (8, 18). To determine the role of MHC class II molecules in the antigen-specific response of immune T lymphocytes to hepatocytes pulsed with HKL, the T cells were cultured in the presence or

TABLE 2. NH_4Cl inhibits the response of immune T cells to hepatocytes pulsed with HKL^a

NH_4Cl	Culture	^3H TdR incorporation (10 ³ cpm; mean $\pm$ SD)	IFN- γ concn (U/ml)
Absent	IT	0.8 $\pm$ 0.2	ND ^b
	IT + HKL-HC	38.3 $\pm$ 10.5 ^c	147.0
Present	IT	0.9 $\pm$ 0.4	ND
	IT + HKL-HC	8.6 $\pm$ 1.9 ^c	10.0

^a Immune T cells (IT) were cultured alone or cocultured with hepatocytes that had been pulsed with HKL (HKL-HC) in the absence or presence of 2 mM NH_4Cl . The results were obtained in a single experiment representative of six experiments.

^b ND, not detectable.

^c Values are significantly different ($P = 0.002$ [Mann-Whitney rank sum test]).

TABLE 3. The optimal response of immune T cells to hepatocytes pulsed with HKL requires cell-cell contact^a

Location of cells		^3H TdR incorporation (10 ³ cpm; mean $\pm$ SD)	Mean IFN- γ concn (U/ml)
Insert	Well		
IT	IT + HKL-HC	344.9 $\pm$ 3.2 ^b	132.0
	HKL-HC	21.3 $\pm$ 7.5 ^b	ND ^c

^a Immune T cells (IT; 6×10^5 per well) and HKL-pulsed hepatocytes (HKL-HC; 6×10^4 per well) in 24-well tissue culture dishes were incubated together or separated by inserts. Values are from triplicate wells in an experiment typical of five experiments. Immune T cells cultured together with either HKL or hepatocytes alone incorporated approximately 18×10^3 cpm of ^3H TdR; IFN- γ was not detectable.

^b Values are significantly different ($P < 0.001$ [Student's *t* test]).

^c ND, not detectable.

absence of monoclonal anti-IA^b antibody. As shown in Table 4, the addition of 10 μg of anti-IA^b per ml reduced T-cell proliferation and the production of IFN- γ more than 10-fold. In contrast, treatment with normal mouse IgG had no effect on the proliferation of immune T cells cultured with HKL-pulsed hepatocytes, while IFN- γ production was reduced only two-fold.

Hepatocytes pulsed with HKL stimulate the response of CD4⁺ immune T lymphocytes preferentially. The MHC class II-restricted response of immune T lymphocytes to macrophages incubated with HKL is confined to the CD4⁺ subpopulation of T cells (8, 18). To identify the responsive T-cell subset(s) in our coculture system, CD4- and CD8-enriched immune T splenocytes were incubated with HKL-pulsed hepatocytes. As shown in Table 5, the total and CD4-enriched immune T-cell populations exhibited 5- to 10-fold increases in ^3H TdR incorporation relative to cells incubated with either hepatocytes or HKL alone. Furthermore, both populations produced elevated levels of IFN- γ when cocultured with antigen-pulsed hepatocytes. Under the same conditions, the CD8-enriched T-cell population incorporated significantly less ^3H TdR and produced little IFN- γ . These findings indicate that hepatocytes pulsed with HKL stimulate preferentially the response of CD4⁺ immune T lymphocytes.

DISCUSSION

An increasing number of observations indicate that rather than serving simply as the targets of effector cells, hepatocytes play an active role in immune responses that occur in the liver. For example, we recently reported that the bulk of *listeriae*

TABLE 4. Monoclonal anti-IA antibody blocks the response of immune T cells to hepatocytes preincubated with HKL^a

Antibody	^3H TdR incorporation (10 ³ cpm; mean $\pm$ SD)	IFN- γ concn (U/ml)
Control	37.7 $\pm$ 7.0	242.0
Anti-IA	3.0 $\pm$ 1.3 ^b	16.0
Normal IgG	39.3 $\pm$ 7.5	109.0

^a Hepatocytes (10^4 cells per well in half-area microtiter plates [Costar]) were preincubated with 10^6 HKL for 4 h in the presence of 10 μg of anti-IA^b monoclonal antibody (BALB/c IgG2a, clone AF6-120.1; Pharmingen) per ml or an equivalent concentration of normal mouse IgG (Cappel). Immune T cells (10^5 per well) were added, and the cells were cocultured for 4 days. The results were obtained from an experiment typical of three similar experiments. Immune T cells incubated with HKL in the absence of hepatocytes incorporated 6,408 cpm of ^3H TdR and produced 13 U of IFN- γ per ml.

^b Significantly less than in either untreated, control cultures or cultures treated with normal mouse IgG ($P < 0.05$ [Student-Newman-Keuls test]).

TABLE 5. Hepatocytes pulsed with HKL stimulate preferentially the biological response of CD4⁺ *Listeria*-immune T lymphocytes

IT culture ^a	[³ H]TdR incorporation (10 ³ cpm; mean ± SD)	IFN-γ concn (U/ml)
Total plus:		
Control	2.1 ± 2.3	ND ^b
HC	12.3 ± 1.4	ND
HKL	14.5 ± 9.0	36.8
HKL-HC	105.2 ± 19.7	234.9
CD4 enriched plus:		
Control	2.2 ± 1.5	ND
HC	22.9 ± 3.7	10.3
HKL	9.1 ± 5.2	16.4
HKL-HC	107.2 ± 21.6	594.5
CD8 enriched plus:		
Control	0.9 ± 0.5	ND
HC	17.3 ± 7.2	ND
HKL	3.8 ± 2.0	ND
HKL-HC	22.8 ± 4.3 ^c	10.0

^a Total, CD4-enriched, and CD8-enriched immune T (IT) cells were incubated for 4 days alone (control) or in the presence of hepatocytes (HC), HKL, or hepatocytes pulsed with HKL (HKL-HC).

^b ND, not detectable.

^c Significantly less than that incorporated by either the total or the CD4-enriched IT population incubated with HKL-HC ($P < 0.05$ [Student-Newman-Keuls test]).

injected intravenously into a mouse and taken up in the liver was associated primarily with the hepatocyte population (8). While *Listeria* injected into immunologically naive mice replicated exponentially within the hepatocytes, *Listeria* injected into immune animals were taken up by hepatocytes and rapidly eliminated. Subsequent *in vitro* studies demonstrated that cytokine-treated hepatocytes had significant antilisterial activity. Hepatocytes incubated in the presence of IFN-γ exhibited a marked decrease in intracellular *Listeria* relative to hepatocytes incubated in its absence (17). The listericidal activity of IFN-γ-treated hepatocytes was diminished by compounds that scavenged or inhibited the production of reactive oxygen, but not nitrogen, intermediates. Taken together, these findings suggest that activation of the oxygen-dependent antimicrobial activity of hepatocytes by cytokines may be an important effector mechanism underlying the elimination of bacteria, including *Listeria*, taken up in the liver.

Other investigators have suggested that hepatocytes might serve as immunomodulating (accessory) cells capable of initiating and perpetuating immune responses occurring in the liver (13, 41). Potential for the productive interaction of T lymphocytes with hepatocytes is indicated by the fact that hepatocytes obtained from biopsies of acutely and chronically inflamed human livers or normal hepatocytes cultured in the presence of IFN-γ exhibit class I and class II MHC molecules (12, 13) and costimulatory adhesion molecules, i.e., ICAM-1 and LFA-3 (1, 28, 39, 40). In addition, hepatocytes can produce a number of soluble costimulatory molecules, including IL-1, IL-6, IL-8, and macrophage and granulocyte-macrophage colony-stimulating factors, which modulate the biological activities of various immune cell types (33–36).

The ability of hepatocytes to serve as antigen-presenting cells and to elicit the antigen-specific response of immune T lymphocytes is supported by the study reported here. The electron micrographs shown in Fig. 1 demonstrate the capacity of murine hepatocytes to internalize HKL. While the mechanism(s) that underlies the internalization of HKL remains to be determined, hepatocytes exhibit cell surface carbohydrate receptors which have been implicated in the binding and up-

take of asialoglycoproteins (2) and apoptotic liver cells (11). Recent studies indicate that these same receptors facilitate the binding and subsequent uptake of viable *Listeria* by a human hepatocarcinoma cell line (HepG2) (9) and by primary cultures of murine hepatocytes (17a). In the latter case, murine hepatocytes treated with anti-asialoglycoprotein receptor antibody or cultured in the presence of elevated concentrations of galactose or mannose exhibited a marked reduction in the number of *Listeria* taken up. These findings suggest that carbohydrate receptors expressed by hepatocytes may also mediate the internalization of HKL.

Murine hepatocytes precultured with HKL stimulated the proliferation of *Listeria*-immune T lymphocytes and the production of IFN-γ. Maximum stimulation was obtained in cocultures that contained 2×10^4 hepatocytes pulsed with 10^6 HKL. The response of immune T cells was reduced significantly in cultures that contained 10^5 hepatocytes. A variety of factors, for example, nutrient deprivation or the production and/or accumulation of metabolic inhibitors such as nitric oxide, could account for the failed response of immune T lymphocytes in coculture. HKL at a higher concentration (5×10^6 per microtiter well) also exerted an inhibitory effect on the biological activity exhibited by immune T cells cocultured with hepatocytes. This observation was not totally unexpected. It is not uncommon in biological systems to find that even highly purified compounds have contrasting effects when tested over a broad range of concentrations. Clearly, one or more components of biochemically complex particles such as HKL could exert concentration-dependent, contradictory effects.

The optimal response of immune T lymphocytes to HKL-pulsed hepatocytes depended upon the expression of class II MHC molecules. Both T-cell proliferation and the production of IFN-γ were reduced markedly when the cells were cocultured in the presence of monoclonal anti-IA antibody. The expression of class II MHC molecules was associated initially with traditional antigen-presenting cells such as macrophages and dendritic cells found in lymphoid tissues. Studies demonstrating the presence of MHC II molecules on the surface of nonlymphoid tissue cells, e.g., fibroblasts (32, 37) and epithelial cells (24), led to the suggestion that such cells could serve in an immunomodulating capacity (38). That is, MHC II-bearing nonlymphoid tissue cells could function as antigen-presenting cells capable of stimulating the antigen-specific response of T lymphocytes. Our findings are in agreement with those published by other investigators who demonstrated the ability of dermal fibroblasts (37), thyroid epithelial cells (24), and epidermal keratinocytes (4) to elicit the antigen-specific, MHC-restricted proliferation of T lymphocytes. Similarly, Franco et al. (13) reported recently that autologous hepatocytes stimulated the MHC-restricted proliferation of CD4⁺ and CD8⁺ T-cell clones derived from the livers of patients with autoimmune chronic active hepatitis.

It is pertinent to note that our evidence implicating MHC class II molecules in antigen presentation by hepatocytes is based upon blocking experiments involving monoclonal anti-IA^b antibody. A high level of nonspecific staining has hampered our efforts to demonstrate (or negate) directly MHC class II antigen expression by hepatocytes cocultured with immune T lymphocytes. In contrast to our experience with cultured cells, freshly isolated mouse hepatocytes did not exhibit excessive background staining and were uniformly MHC class II negative by immunohistochemical analysis (unpublished observation).

As a general rule, macrophages utilize two distinct pathways to generate the antigenic determinants presented to T cells (30). Antigens derived from an exogenous source are degraded

within the lysosomal compartment, and immunogenic peptides are presented in association with MHC class II molecules to CD4⁺ T lymphocytes. Immunogenic peptides synthesized endogenously, on the other hand, are presented in association with class I MHC molecules to CD8⁺ T lymphocytes. The MHC class II-restricted response of *Listeria*-immune T lymphocytes to hepatocytes pulsed with HKL correlates with the preferential stimulation of CD4⁺ T lymphocytes shown in Table 5. This result concurs with the findings of others demonstrating that CD4⁺ (but not CD8⁺) *Listeria*-immune T lymphocytes respond to macrophages pulsed with HKL in a MHC class II-restricted manner (8, 18).

The role of CD4⁺ T cells in host defenses against listeriae remains to be fully delineated. While it is generally agreed that CD8⁺ T lymphocytes play the predominant role in protective immunity to listerial infection, CD4⁺ T lymphocytes have been implicated in delayed-type hypersensitivity to listerial antigens, granulomatous formation, IFN- γ production, and resistance to listerial infections (3, 20–22, 27). Undoubtedly, a large number of dead organisms are produced during the course and ultimate resolution of listerial infections of the liver. Our results suggest that hepatocytes may process and present epitopes derived from these dead organisms and stimulate the proliferation and production of IFN- γ by sensitized CD4⁺ T lymphocytes infiltrating the livers of *Listeria*-infected mice.

ACKNOWLEDGMENTS

We acknowledge Athanasia Sagnimeni, Timothy Dean Ryan, and Anne Chapas for their excellent technical assistance and Artie Reis (Department of Pathology, University of Pittsburgh Medical Center) for the electron micrograph shown in this study.

This research was supported by Public Health Service grants AI24141 and DK44367 from the National Institutes of Health.

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