INFECTION AND IMMUNITY, July 1990, p. 2313–2319
0019-9567/90/072313-07$02.00/0

Vol. 58, No. 7

Accessory Function of Kupffer Cells in the Antigen-Specific Blastogenic Response of an L3T4$^+$ T-Lymphocyte Clone to *Listeria monocytogenes*

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Received 11 January 1990/Accepted 13 April 1990

The function of Kupffer cells in the development of protective immunity to infection by *Listeria monocytogenes* is controversial. To determine their role in antilisterial host defenses, Kupffer cells were separated from other nonparenchymal cells of the liver by centrifugation on a metrizamide gradient followed by adherence to glass or plastic. The resultant highly enriched Kupffer cell population supported the antigen-specific blastogenic response ([^{3}H]thymidine incorporation) of cloned L3T4$^+$ T lymphocytes to *L. monocytogenes* in vitro. Blastogenesis was dependent upon the duration of the incubation period, the concentration of the antigen, and the number of Kupffer cells in culture. Maximum reactivity was greater than that observed when the same T-cell population was incubated with adherent peritoneal exudate cells and antigen under optimal conditions. The addition of antibodies specific for murine interleukin-1β to cocultures of Kupffer cells and T lymphocytes eliminated the antigen-stimulated incorporation of [^{3}H]thymidine, indicating a requirement for interleukin-1. Analysis of the culture supernatants demonstrated that, in addition to interleukin-1, granulocyte-macrophage colony-stimulating factor, interleukin-6, and gamma interferon were elaborated in cocultures containing cloned T lymphocytes, Kupffer cells, and antigen. These results suggest that Kupffer cells may serve a critical role in the development of immunity to infection by *L. monocytogenes* in vivo.

Listeriosis in mice is a widely used prototypic model for the development of protective immunity to infections by facultative intracellular pathogens (8). After the intravenous injection of nonimmune animals with a sublethal dose of *Listeria monocytogenes*, the bulk of the microorganisms are rapidly cleared from the blood by fixed tissue macrophage cells (Kupffer cells), which line the liver sinusoids (18, 19). After an initial period of decline, the number of *L. monocytogenes* increases exponentially as the organisms proliferate within phagocytic cells. Recovery from a primary infection and immunity to subsequent infection are mediated by antigen-specific T-lymphocyte populations (5, 27). This is best illustrated by the capacity of sensitized T lymphocytes derived from mice immune to *L. monocytogenes* to confer resistance to nonimmune animals (14, 20). The precise mechanisms underlying the protection conferred by sensitized T lymphocytes is unknown; it is presumed, however, that the synthesis and secretion of lymphokines are major factors. This presumption is supported by recent experimental evidence demonstrating the significant role of gamma interferon and colony-stimulating factors secreted by cloned L3T4$^+$ T lymphocytes sensitized to *L. monocytogenes* in the transfer of resistance to nonimmune mice (20, 22).

Although it is accepted that the specificity of the immune response to infection by *L. monocytogenes* resides in the T-lymphocyte population, the actual elimination of the organism from infected animals is a function of cells of mononuclear phagocyte lineage. Thus, the eventual sterilization of the livers of animals injected intravenously with low doses of *L. monocytogenes* correlates with the influx of blood monocytes and the subsequent congregation of activated macrophage cells in infectious foci, which initially contain proliferating microorganisms (18, 19, 26, 29).

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Although the bulk of *L. monocytogenes* injected intravenously into mice is rapidly cleared from the blood by Kupffer cells, the role of Kupffer cells in the development of immunity is poorly understood. In fact, it has been suggested that Kupffer cells play an insignificant role in the development of resistance during a primary listerial infection (26). Our data suggest, however, that this mononuclear phagocyte population may be an important factor in the immune response to *L. monocytogenes*. Here, we report for the first time that purified Kupffer cells induce the antigen-dependent proliferation of *L. monocytogenes*-specific T lymphocytes and secrete a number of soluble factors capable of influencing the course of infection.

MATERIALS AND METHODS

Animals. Female C57BL/6J mice, purchased from Jackson Laboratories, Bar Harbor, Maine, were housed in accordance with the guidelines set forth by the Institute of Laboratory Animals Resources, National Research Council. Animals between 6 and 16 weeks of age served as the source of cells used in the experiments described herein.

Bacteria. *L. monocytogenes* (EGD strain) was cultured and maintained as previously described (31). Heat-killed *L. monocytogenes* (HKL) were prepared by incubating bacterial suspensions for 1 h at 60°C. HKL were washed twice, suspended in saline at 10^9 cells per ml, and frozen at −70°C.

Kupffer cells. Murine Kupffer cells were obtained and cultured by a modification of techniques described previously (28). The superior mesenteric veins of anesthetized mice were cannulated, and the livers were perfused slowly with 20 ml of Hanks balanced salt solution (HBSS) containing 5% heat-inactivated fetal bovine serum (FBS; Sterile Systems, Inc., Logan, Utah), 100 U of collagenase (type IV; Sigma Chemical Co., St. Louis, Mo.) per ml, and 0.001% DNase I (type I; Sigma) at room temperature. Once perfu-

sion was established, the portal veins were severed, allowing efflux of the perfusate. Perfused livers were excised aseptically, transferred to polystyrene petri plates containing FBS, collagenase, and DNase in HBSS, and incubated at room temperature for approximately 1 h. The gall bladders and extraneous tissues were removed, the livers were teased apart with curved forceps over an 80-mesh stainless steel screen, and the teased cells were flushed through the screen with ice-cold HBSS containing FBS, collagenase, and DNase. The resulting cell suspension was centrifuged twice at 30 × *g* for 4 min at 4°C to remove parenchymal cells. The cells that remained in suspension were pelleted by centrifugation at 400 × *g* for 10 min at 4°C, suspended in 5 ml of ice-cold HBSS (total volume), and mixed with 7 ml of cold 30% metrizamide (Sigma) in HBSS. The mixture was overlaid with 3 ml of cold HBSS and centrifuged at 1,500 × *g* for 20 min at 4°C. The nonparenchymal cells banding at the HBSS-metrizamide interface were collected and washed by centrifugation three times at 400 × *g* for 10 min at 4°C in RPMI 1640 (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 10% FBS, 100 U of penicillin per ml, and 100 μg of streptomycin per ml (medium). The cells in the final pellet were suspended, counted, diluted in medium to the concentration noted below, and aliquoted into 96-well polystyrene tissue culture plates or into 24-well plates that contained sterile, 1.6-cm glass cover slips. The plates were centrifuged at 250 × *g* for 5 min at room temperature and then incubated for 15 min at 37°C in a humidified environment composed of 5% CO_2 in air. The nonadherent cells were removed by washing the monolayer six times with warm medium; washed cover slips were transferred to fresh multiwell plates. The adherent, Kupffer cell-enriched population was incubated further under the conditions described below.

Peritoneal exudate cells. The peritoneal cells were harvested from mice injected intraperitoneally with 60 μg of concanavalin A (ConA) in 1 ml of saline 3 days previously (3, 30). The cells were washed once, suspended in medium, counted, and seeded into 96-well tissue culture plates or 24-well plates containing glass cover slips. The plates were incubated for 2 h at 37°C in an humidified environment containing 5% CO_2 in air to allow the cells to attach. At the termination of the incubation period, the cell monolayers were washed six times with warm medium. The remaining adherent cells in the 96-well plates were cultured under the conditions described below. Cells adherent to cover slips were fixed and stored at 4°C in 0.5% paraformaldehyde in HBSS.

T-lymphocyte clone. The production and characterization of the 8D7 T-cell clone have been reported previously (20, 22). 8D7 T lymphocytes respond specifically to *L. monocytogenes* in a dose-dependent manner and are of the Thy-1.2$^+$ L3T4$^+$ Lyt-2$^-$ phenotype. To propagate the 8D7 clone, 2.5 × 10^5 cells per ml were subcultured every 2 weeks in fresh RPMI 1640 medium containing 10% heat-inactivated FBS, 1 mM sodium pyruvate, 1 mM L-glutamine, 5 × 10^{-5} M 2-mercaptoethanol, 100 U of penicillin per ml, and 100 μg of streptomycin per ml (cloning medium). In addition, cell cultures contained 5 × 10^5 irradiated (1.9 Gy) mouse splenocytes per ml, 5 × 10^6 HKL/ml, and 20% supernatant derived from ConA-stimulated rat splenocyte cultures, which served as an exogenous source of interleukin-2 (IL-2). Fresh cloning medium containing 20% rat IL-2 was added to the cultures twice a week.

T-lymphocyte blastogenesis. At the end of a subculture period, 8D7 T lymphocytes, having received neither fresh feeder cells nor additional antigen during the preceding 2 weeks, were separated from nonviable feeder cells and other cell debris by centrifugation on Lympholyte M cell separation medium (Accurate Chemical Co., Westbury, N.Y.). To analyze the blastogenic capacity of this purified population, 5 × 10^4 T lymphocytes in 0.2 ml of cloning medium per well were seeded into 96-well plates and either cultured alone or cocultured with Kupffer cells or adherent peritoneal exudate cells and various concentrations of HKL. The cells were pulsed with 1 μCi of [*methyl*-^{3}H]thymidine (6.7 Ci/mmol; ICN Radiochemicals, Irvine, Calif.) per well during the last 4 or 18 h of culture. At the termination of the culture period, the cells were collected onto glass fiber filters with a semiautomated harvester. The incorporation of radiolabeled thymidine into cells was assessed by liquid scintillation counting.

Immunofluorescence analyses of cell surface antigens. Adherent nonparenchymal cells expressing Mac-1 antigens, Ia^b antigens, or coagulation factor VIII-associated protein were quantified by indirect immunofluorescence analysis as described previously (2). Cells fixed to 1.6-cm cover slips with 0.5% paraformaldehyde were incubated with monoclonal rat immunoglobulin G2b (IgG2b) anti-human/murine Mac-1 (10 μg/ml in HBSS; Boehringer Mannheim Biochemicals, Indianapolis, Ind.), monoclonal mouse IgM anti-Ia^b (100 μg/ml in HBSS; Bioproducts for Science Inc., Indianapolis, Ind.), or polyclonal rabbit anti-human coagulation factor VIII-associated protein (100 μg/ml in HBSS; Calbiochem Corp., San Diego, Calif.). After incubation for 0.5 h at 4°C, the cover slips were washed in HBSS and incubated under the same conditions with the fluorescein-conjugated F(ab')$_2$ fragment of either goat anti-rat IgG, goat anti-mouse IgM (heavy chain specific), or sheep anti-rabbit IgG (Organon Teknika-Cappel, West Chester, Pa.) for the analyses of Mac-1, Ia^b, and factor VIII-associated protein, respectively. The cover slips were subsequently washed and examined by fluorescence microscopy. The percentage of antigen-positive cells was calculated from the number of fluorescent cells in a population of 200 cells counted.

Cytochemistry. Cytochemical analysis of peroxidase activity was performed by a modification (24) of the method originally described by L. S. Kaplow; analysis of nonspecific esterase activity was performed by the method of Koski et al. (12). In both assays, the percentage of cells possessing enzyme activity was calculated from the number of cells exhibiting a visible precipitate among a population of 200 cells counted. Less than 0.5% of cells incubated in reaction mixtures without substrate exhibited precipitate.

Fc receptor analysis. The percentage of cells expressing receptors for the Fc portion of IgG was determined by erythrocyte rosetting techniques described previously (4). Briefly, washed sheep erythrocytes (sRBC) were incubated for 15 min at 37°C with an equal volume of rabbit anti-sRBC IgG (1:500 dilution; Cordis Laboratories, Miami, Fla.), washed, and suspended at 0.5% (vol/vol) in Dulbecco modified Eagle medium (GIBCO). To assess Fc receptor expression, enriched Kupffer cell populations bound to glass cover slips were incubated for 0.5 h at 37°C with 1 ml of IgG-coated sRBC, washed, and examined by light microscopy. The percentage of receptor-positive cells was calculated from the number of cells distinguished by three or more bound sRBC in a population of 200 cells counted. Cells incubated with uncoated sRBC served as the control for binding not mediated by receptors specific for IgG.

Cytokine production and analysis. Kupffer cells derived from 1 × 10^6 nonparenchymal cells seeded into cover slips

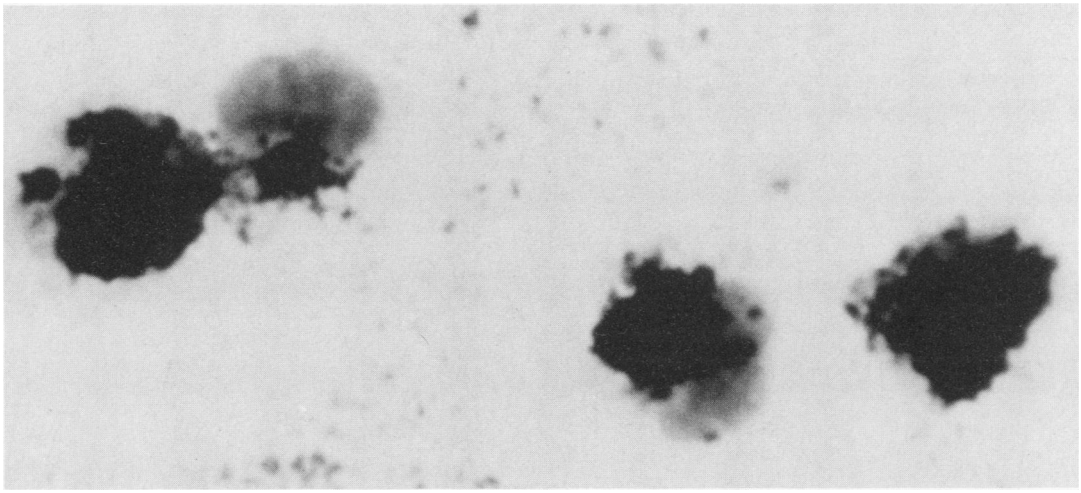

FIG. 1. Adherent nonparenchymal cell population. Nonparenchymal cells (10^6 cells in 2 ml of RPMI 1640 supplemented with 20% FBS) were seeded into multiwelled plates containing 1.6-cm cover slips. The plates were centrifuged and incubated for 15 min at 37°C. The cover slips were subsequently washed, and the adherent cells were incubated for 45 min at 37°C with a 0.2% suspension of carbon particles in RPMI 1640 containing 10% FBS. The cell monolayers were washed, fixed with methanol, and stained with Giemsa stain. Magnification, ×400.

and 5×10^5 cloned T lymphocytes were either cultured separately or cocultured for 2 days at 37°C in 2 ml of cloning medium with or without 1×10^7 HKL. At the termination of the incubation period, the culture supernatants were collected, filter sterilized, and stored at −20°C until analyzed for the presence of the cytokines enumerated below.

Tumor necrosis factor was quantitated by a modification of an assay described previously, using the metabolism of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide as a measure of cell viability (23, 25). Wells of flat-bottom microdilution plates were inoculated with 5×10^4 L-929 mouse fibroblasts in 100 μl of RPMI 1640 medium supplemented with 10% FBS and antibiotics, and the plates were incubated overnight at 37°C in a humidified atmosphere composed of 5% CO_2 in air. On the following day, serial dilutions of each culture supernatant were prepared in the wells. Actinomycin D (1 μg/ml) was then added, and the plates were incubated for 24 h at 37°C. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (20 μl of a 5-mg/ml solution in phosphate-buffered saline) was added to each well for the final 4 h of culture. To dissolve the dark blue formazan crystals produced by viable, intact cells, 100 μl of 10% sodium dodecyl sulfate in 0.01 N HCl was added to each well, and the plates were incubated at 37°C overnight. Cell lysis was quantitated on a microELISA reader with a 570-nm test filter and a 630-nm reference filter.

Interferon (IFN) was measured by a modification of the plaque inhibition assay (1). Supernatants were serially diluted in microdilution plates, and 2.5×10^4 L-929 cells were added to each well. After an overnight incubation period, encephalomyocarditis virus was added to the wells, and the plates were cultured for an additional 24 h. Each well received 20 μl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (5 mg/ml in phosphate-buffered saline) during the last 4 h of the culture period. The plates were then processed as described above for the tumor necrosis factor assay.

IL-1 activity was assessed by using the D10.G4.1 bioassay described previously (13). IL-6 (IFN-β_2) activity was determined by using the IL-6-dependent murine B cell hybridoma B9 1B (9). The presence of either IL-2 or IL-4 in culture supernatants was assessed by the ability of samples to support the growth of the factor-dependent cell line CTLL-2 (7). IL-3 and granulocyte-macrophage colony-stimulating factor (GM-CSF) were quantitated using the growth factor-dependent cell lines FDCP-1 and 32D cl 3 as described previously (20, 21).

The following cytokines served as standards in the analyses undertaken: recombinant human tumor necrosis factor (Genentech, Inc., South San Francisco, Calif.); recombinant murine IFN-γ (Genentech); recombinant human IL-1α (Steven Gillis, Immunex Corp., Seattle, Wash.); recombinant human IL-6 (Genzyme Corp., Boston, Mass.); IL-2 generated in ConA-stimulated rat splenocyte cultures; and IL-3 contained in WEHI cell-conditioned culture medium. Antibody to murine IFN-γ was prepared from the ascites fluid of pristane-treated outbred athymic nude mice (Harlan Sprague-Dawley, Inc., Indianapolis, Ind.) injected with 2×10^7 R4-6A2 hybridoma cells (America Type Culture Collection, Rockville, Md.) (22). In all of the cases above, the titers of culture supernatants were determined in triplicate, and the units of cytokine activity per milliliter of supernatant was calculated from the results. One unit of activity is defined as the amount required to elicit a half-maximal effect. The values were obtained in two independent determinations.

Statistics. Data are expressed as the means ± standard deviations. Individual means were compared by using the nonpaired Student *t* test.

RESULTS

Characterization of the adherent nonparenchymal cells. Treatment of livers under the conditions described in Materials and Methods yielded approximately 10^7 nonparenchymal cells (92 to 94% viable) per liver. To determine the percentage of Kupffer cells in this nonparenchymal cell population, some mice were injected intravenously with a 10% solution of India ink in HBSS 2 h before cell harvest. The percentage of cells containing carbon particles, correlating with the percentage of Kupffer cells in the nonparenchymal cell population, varied between 34 and 37%. A photomicrograph of the adherent cells derived from the nonparenchymal cell population is shown in Fig. 1. The adherent cell population consisted, for the most part, of large

TABLE 1. Characteristics of adherent nonparenchymal cells

Characteristic	% of cells[a] ± SD
Nonspecific esterase positive	94 ± 2
Peroxidase positive	71 ± 4
Phagocytic[b]	84 ± 3
Ia[b] antigen positive	48 ± 7[c]
IgG Fc receptor positive[d]	89 ± 3
Mac-1 antigen positive	1 ± 1[e]
Factor VIII positive	<0.5[f]

[a] Data are expressed in terms of the mean percentage of cells exhibiting a specific trait calculated from two or more determinations.

[b] Cells capable of internalizing latex beads. Cells were incubated for 0.5 h at 37°C with a 0.2% suspension of latex beads (1.1-μm diameter; Sigma), washed, and examined by phase-contrast microscopy.

[c] Approximately 2% of cells incubated with the fluorescein-labeled, secondary antibody only were positive.

[d] Approximately 3% of cells incubated with uncoated sRBC formed rosettes.

[e] Adherent peritoneal exudate cells stained simultaneously were approximately 70% Mac-1 antigen positive.

[f] The total nonparenchymal cell fraction harvested from the metrizamide gradient was approximately 28% antigen positive.

phagocytic cells. The vast majority of these cells were Kupffer cells as judged by a number of criteria (Table 1). The bulk of the cells exhibited nonspecific esterase activity, peroxidase activity, and the capacity to internalize carbon particles or latex beads. In addition, a large percentage of the cells expressed Ia[b] antigens and cell surface receptors for the Fc portion of IgG. In contrast, only a negligible percentage of cells expressed either Mac-1 or coagulation factor VIII antigens, which are associated with contaminating peripheral blood monocytes (16, 29) and endothelial cells (10), respectively. Finally, the adherent nonparenchymal cell population failed to produce either hydrogen peroxide or superoxide anions in response to treatment with zymosan or phorbol myristate acetate (PMA) (data not shown). This finding correlates with the results of others who reported that, relative to other mononuclear phagocyte populations, Kupffer cells are deficient in their capacity to generate reactive oxygen metabolites (17).

Antigen-specific T-cell blastogenesis. Experiments were undertaken to determine whether Kupffer cells could serve as accessory cells in the antigen-dependent proliferation of T lymphocytes to *L. monocytogenes*. The results demonstrated the capacity of Kupffer cells to support the blastogenic response of the 8D7 T-cell clone to HKL. Blastogenesis, measured in terms of [^{3}H]thymidine uptake, was dependent upon HKL concentration and the number of Kupffer cells per well (Fig. 2). Maximum uptake of [^{3}H]thymidine occurred when 5×10^4 8D7 T lymphocytes were cultured with 1×10^6 HKL in wells that had been inoculated with 1×10^5 nonparenchymal cells. Kupffer cells or 8D7 clone T cells cultured alone, with or without antigen, incorporated counts only slightly above background.

Kinetics of T-cell blastogenesis. The maximum blastogenic response of 8D7 T cells to HKL was observed after T lymphocytes and Kupffer cells were cocultured for 3 days (Fig. 3). [^{3}H]thymidine incorporation decreased sharply over the next 24 h and then diminished more gradually throughout the remainder of an 8-day incubation period. The antigen-specific blastogenic response of T lymphocytes cocultured with peritoneal exudate cells exhibited similar kinetics. However, the maximum response with peritoneal exudate cells was less than that observed with Kupffer cells. Antigen-stimulated [^{3}H]thymidine incorporation by T lymphocytes was completely abolished when the cells were cocultured with 5×10^4 or 2×10^5 peritoneal exudate cells seeded into a well (data not shown).

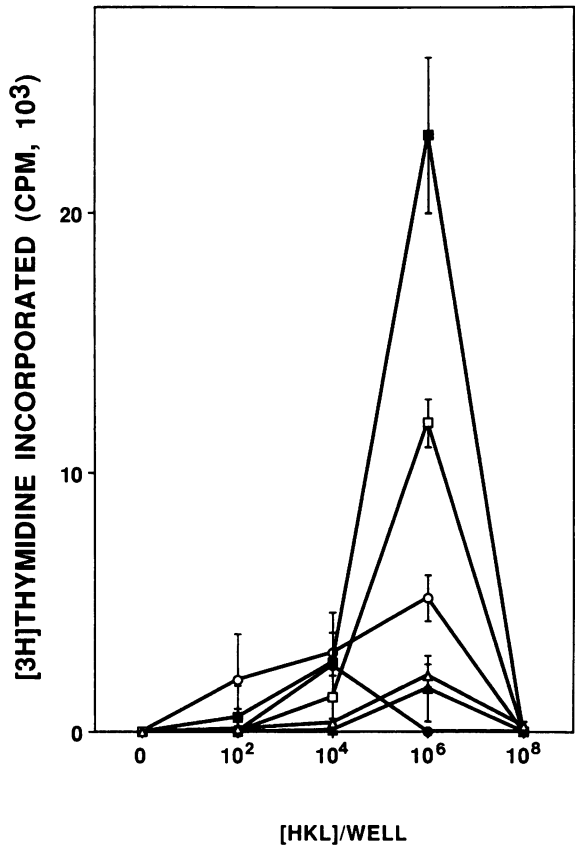

FIG. 2. Blastogenesis of *L. monocytogenes*-specific T lymphocytes is influenced by antigen concentration and Kupffer cell density. Wells of 96-well plates were uninoculated or inoculated with 4×10^3 (△), 1×10^4 (▲), 4×10^4 (□), 1×10^5 (■), 4×10^5 (○), or 1×10^6 (●) nonparenchymal cells. The plates were centrifuged, incubated for 15 min to allow cell attachment, and washed to remove the nonadherent cells. Then 5×10^4 8D7 T lymphocytes with or without increasing concentrations of HKL in 0.2 ml (final volume) of cloning medium were added to the wells, and the plates were incubated for 3 days; each well received 1 μCi of [^{3}H]thymidine during the last 18 h of the culture period. The data were derived from a single experiment that was representative of four similar experiments. Values are the means ± standard deviations of counts per minute incorporated by the HKL-stimulated cells in three identical wells corrected by subtracting the counts incorporated by T cells and Kupffer cells cocultured in the absence of antigen. Kupffer cells or T cells cultured alone with antigen incorporated approximately 600 and 1,400 cpm, respectively.

Anti-IL-1β inhibits blastogenesis. The blastogenic response of 8D7 T lymphocytes to HKL was dependent upon the presence of IL-1β. Inclusion of polyclonal rabbit anti-murine IL-1β serum in the culture medium abrogated the increase in [^{3}H]thymidine uptake observed when HKL were added to cocultures of 8D7 T lymphocytes and Kupffer cells (Table 2).

Soluble factors elaborated in cocultures of 8D7 T lymphocytes and Kupffer cells. The supernatant fluids obtained from cocultures of 8D7 T lymphocytes and Kupffer cells contained soluble factors capable of influencing T-lymphocyte blastogenesis and the course of listerial infection in vivo. As one would expect based upon the results of the experiment above, IL-1 was present in the supernatants derived from

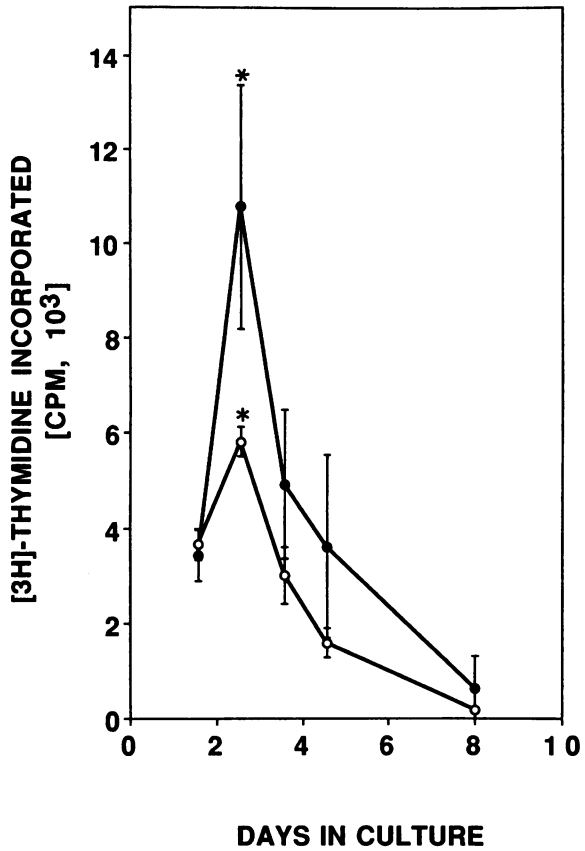

FIG. 3. Kinetics of the proliferative response of 8D7 T lymphocytes to HKL. Individual wells of 96-well plates were inoculated with 10^5 nonparenchymal cells (●) or 10^4 peritoneal exudate cells (○). The plates were incubated to allow cell attachment and washed to remove the nonadherent cells as described in Materials and Methods. Then 5×10^4 *L. monocytogenes*-specific T lymphocytes and/or 10^6 HKL in 0.2 ml (total volume) of cloning medium were added to the appropriate wells, and the plates were incubated for the times indicated; each well received 1 μCi of [^{3}H]thymidine during the last 4 h of the culture period. The data were obtained from a single experiment representative of three similar experiments. Values are the means ± standard deviations of counts per minute incorporated by cells in three identical wells corrected by subtracting the counts incorporated by T cells and accessory cell populations cocultured in the absence of antigen. Counts incorporated by Kupffer cells or peritoneal exudate cells incubated alone with or without antigen approximated the background counts per well (300 to 800 cpm). *, Significantly different ($P < 0.005$).

cocultures of cells incubated with antigen (Table 3). In addition, the activities of IL-6 and IFN were elevated in T lymphocyte-Kupffer cell cocultures relative to cultures of either cell population incubated alone. GM-CSF and IFN activities were enhanced, albeit slightly, by the presence of HKL in the culture system.

DISCUSSION

Fixed tissue macrophage cells in the liver (Kupffer cells) are a major factor in blood filtration and in the clearance of blood-borne pathogens. Using listeriosis as an experimental model, investigators have found that greater that 60% of the microorganisms in a sublethal dose of *L. monocytogenes* administered to a mouse intravenously are recovered in the liver within 10 min after injection (18, 19). After an initial decline, the number of viable organisms in the liver increases exponentially over the next 2 to 3 days (18, 19). Death of the microorganisms and eventual sterilization of the liver correlate with the appearance of monocytes immigrating in and around infective foci (18, 19, 22). These findings have led other investigators to suggest that Kupffer cells fail to make a significant contribution to resistance during a primary *Listeria* infection (26). This suggestion is supported by experiments that demonstrate the diminished capacity of Kupffer cells to generate reactive oxygen metabolites, i.e., hydrogen peroxide and superoxide anion, and to kill intracellular pathogens (17, 18, 26).

TABLE 2. IL-1β-dependent blastogenesis of 8D7 T lymphocytes[a]

Addition to culture	Kupffer cells[b]	
	−	+
None	357 ± 88	782 ± 119
HKL	426 ± 156	1,291 ± 594
8D7 T cells	665 ± 486	4,370 ± 1,020
8D7 T cells + HKL	774 ± 531	9,498 ± 1,472[c]
8D7 T cells + HKL + anti-IL-1β[d]	ND[e]	4,476 ± 770[c]

[a] 8D7 T lymphocytes (5×10^4/0.2 ml of cloning medium with or without 10^6 HKL per 6-mm well) were cultured alone or cocultured with Kupffer cells for 3 days at 37°C. Each well received 1 μCi of [^{3}H]thymidine during the last 18 h of the culture period. The values, representing the mean counts per minute incorporated by cells in three identical wells, were obtained in a representative experiment.

[b] Micro-dilution wells were previously uninoculated or contained the Kupffer cell population derived from 10^5 nonparenchymal cells seeded per well and processed as described in Materials and Methods.

[c] Significantly different ($P < 0.005$).

[d] Polyclonal rabbit anti-mouse IL-1β was the generous gift of Robert C. Newton, DuPont Laboratory, Glenolden, Pa. Each well received a final 1:2,560 dilution of antiserum capable of neutralizing approximately 2,000 half-maximal units of murine IL-1β in the D10 bioassay.

[e] ND, Not determined.

Our results indicate the Kupffer cells exhibit a number of biological functions that are crucial to the development and expression of protective immunity to *L. monocytogenes*. Most importantly, we demonstrated for the first time that Kupffer cell are capable of promoting the *L. monocytogenes*-specific blastogenic response of L3T4$^+$ T lymphocytes. The magnitude of this response was dependent upon Kupffer cell density, antigen (HKL) concentration, and time in culture. Maximum [^{3}H]thymidine incorporation in the presence of antigen was observed when 5×10^4 T lymphocytes were cocultured with the adherent (Kupffer) cell population derived from 1×10^5 nonparenchymal cells. 8D7 T lymphocytes incubated with antigen and larger numbers of Kupffer cells exhibited diminished blastogenic activity. This observation is in agreement with the previous findings of others, who reported that Kupffer cells present at high cell density suppress T-cell proliferation (28). We also found that the proliferative response to HKL was abrogated in cocultures containing a dense population of peritoneal macrophage cells, suggesting that the suppression of T-cell proliferation by a dense population of accessory cells is not a biological activity unique to Kupffer cells. In addition, the proliferative response of 8D7 T lymphocytes to antigen was greater in cocultures that contained Kupffer cells than in those that contained an optimal number of peritoneal macrophage cells. This occurred in spite of the fact that, as in the case of Kupffer cells reported here, a relatively high percentage (40 to 80%) of peritoneal macrophages elicited by intraperitoneal injection of ConA express cell surface Ia antigens (3). This finding suggests that Kupffer cells may be particularly well suited to stimulating T cell-mediated immunity.

TABLE 3. Soluble factors secreted by 8D7 T lymphocytes and Kupffer cells in culture[a]

Factor	U/ml					
	KC	KC + HKL	KC + 8D7	KC + 8D7 + HKL	87D + HKL	8D7
IL-1	<10	40	20	80	<10	<10
TNF[b]	<20	<20	<20	<20	<20	<20
IL-2/IL-4	<10	<10	<10	<10	<10	<10
IL-3	<20	<20	<20	<20	<20	<20
IL-6	40	320	640	640	<20	<20
M-CSF[c]	34	36	47	33	2	25
GM-CSF	<20	<20	<20	20	<20	<20
IFN	<10	10	160(160)[d]	320(160)[d]	<10	<10

[a] Cytokine activity (half-maximal units per milliliter) in the supernatants obtained from cultures of Kupffer cells (KC) adherent to glass cover slips seeded with 1×10^6 nonparenchymal cells and/or of 5×10^5 8D7 T lymphocytes incubated alone or in combination for 2 days in the absence or the presence of 10^7 HKL.
[b] TNF, Tumor necrosis factor.
[c] M-CSF, Macrophage CSF was quantitated directly by a double-antibody radioimmunoassay (31). One unit stimulates the formation of one colony in a standard bone marrow cell colony forming assay. The background, i.e., medium without cells, was 14 U/ml.
[d] Numbers within parentheses indicate IFN activities in supernatants pretreated with 3,000 nU of anti-murine IFN-γ per ml.

Kupffer cells and 8D7 T lymphocytes cocultured in the presence of antigen synthesized and secreted a number of soluble factors that are potentially important as modulators of the immune response to *L. monocytogenes*. Of these, IL-1β, a cytokine of mononuclear phagocyte origin (11, 15), was required for the antigen-stimulated proliferation of the 8D7 T-lymphocyte clone. Polyclonal rabbit anti-mouse IL-1β serum added to the culture medium reduced the level of DNA synthesis to that observed when 8D7 T cells and Kupffer cells were cocultured in the absence of antigen. This finding is in agreement with the results of others, who reported that the proliferative response of an *L. monocytogenes*-specific T-cell clone to antigen-pulsed peritoneal exudate cells was abolished by the addition of antiserum specific for IL-1 (13).

IL-6 was present in the supernatants obtained from all the cultures that contained Kupffer cells. The content of IL-6 in these cultures was enhanced significantly by the inclusion of HKL or 8D7 T cells. 8D7 T lymphocytes cultured alone for 2 days with HKL or with 2.5 μg of ConA per ml (data not shown) failed to produce IL-6, suggesting that the IL-6 detected in the coculture supernatants was produced by Kupffer cells. IL-6, known alternatively as IFN-β_2, B-cell stimulatory factor type 2, and hepatocyte stimulatory factor, exhibits a number of biological activities that are potentially important in the immune response to *L. monocytogenes* (32). IL-6 synthesized by Kupffer cells could, for example, stimulate the proliferation of activated T cells, promote the production of antibody by activated B cells, and enhance the synthesis of acute-phase proteins by hepatocytes.

The supernatant obtained from cocultures of Kupffer cells and 8D7 T lymphocytes incubated in the absence of antigen contained a significant amount of IFN activity. This activity was not neutralized by antibody specific for IFN-γ, which is synthesized by T lymphocytes. This finding suggests that the IFN detected is of Kupffer cell origin and that 8D7 T lymphocytes are capable of stimulating IFN synthesis by Kupffer cells. The production of IFN by Kupffer cells was confirmed by the results of the IL-6 (IFN-β_2) assay. The presence of HKL during the incubation period resulted in a slight increase in the amount of IFN activity that could subsequently be detected in the coculture supernatants. This increase was nullified by treatment of the supernatants with antibody specific for IFN-γ. Thus, the increase in IFN activity detected was probably due to IFN-γ produced by antigen-stimulated 8D7 T lymphocytes. The production of IFN-γ by 8D7 T lymphocytes incubated with irradiated mouse splenocytes and HKL or with 2.5 μg of ConA per ml has been demonstrated previously (20; unpublished observation).

The antigen-specific production of GM-CSF by 8D7 T lymphocytes confirms our previous reports demonstrating the elevated production of CSFs by both immune splenocytes and cloned T lymphocytes, including the 8D7 clone, incubated with HKL (20, 21). In the studies detailed in these reports, maximum values for GM-CSF and IL-3 were assessed only when specific antibody was used to neutralize the IFN-γ also present in the culture supernatants. The presence of IFN-β_2 and the unavailability of the appropriate antibody to block its activity could account for the relatively small amount of GM-CSF, as well as the absence of IL-3, detectable in the culture supernatants analyzed here. Nevertheless, the increased synthesis of CSFs by antigen-stimulated $L3T4^+$ T lymphocytes could influence the immunological response to *L. monocytogenes* during infection by enhancing both the production and the biological activity of mononuclear phagocytes migrating into infective foci as previously suggested (20, 21, 31).

ACKNOWLEDGMENTS

We gratefully acknowledge Lisa Garbin, Lisa Barczynski, Reina Palko, and Scott Nolan for their fine technical assistance, Alan Winkelstein for his helpful discussion, and Patricia Vasilatos for her aid in preparation of this manuscript.

This research was supported by Public Health Service grant AI 24141 from the National Institutes of Health.

LITERATURE CITED

1. **Armstrong, J. A.** 1971. Semi-micro, dye-binding assay for rabbit interferon. Appl. Microbiol. **21**:723–725.
2. **Beller, D. I., J.-M. Kiely, and E. R. Unanue.** 1980. Regulation of macrophage populations. I. Preferential induction of Ia-rich peritoneal exudates by immunologic stimuli. J. Immunol. **124**: 1426–1440.
3. **Beller, D. I., and E. R. Unanue.** 1981. Regulation of macrophage populations. II. Synthesis and expression of Ia antigens by peritoneal exudate macrophages is a transient event. J. Immunol. **126**:263–269.
4. **Bianco, C., and B. Pytowski.** 1981. Fc and C3 receptors, p. 273–282. *In* D. O. Adams, P. J. Edelson, and H. Koren (ed.), Methods for studying mononuclear phagocytes. Academic Press, Inc., New York.
5. **Blanden, R. V., and R. E. Langman.** 1972. Cell-mediated immunity to bacterial infection in the mouse. Thymus-derived cells as effectors of acquired resistance to *Listeria monocytogenes*. Scand. J. Immunol. **1**:379–391.

6. **Boegel, F., A. Waheed, and R. K. Shadduck.** 1981. Evaluation of radioimmunoassay and *in vitro* colony assay techniques for determination of colony-stimulating factor and inhibitory activity in murine serum and tissue. Blood **58**:1141–1147.
7. **Gillis, S., M. M. Ferm, W. Ou, and K. A. Smith.** 1978. T cell growth factor: parameters of production and quantitative microassay for activity. J. Immunol. **120**:2027–2032.
8. **Hahn, H., and S. H. E. Kaufmann.** 1981. The role of cell-mediated immunity in bacterial infections. Rev. Infect. Dis. **3**:1221–1250.
9. **Helle, M., L. Boeije, and L. Arden.** 1988. Functional discrimination interleukin 6 and interleukin 1. Eur. J. Immunol. **18**: 1535–1540.
10. **Jaffe, E. A., L. W. Hoyer, R. L. Nachman.** 1973. Synthesis of antihemophilic factor antigen by cultured human endothelial cells. J. Clin. Invest. **53**:2757–2764.
11. **Koide, S. L., and R. M. Steinman.** 1987. Induction of murine interleukin-1: stimuli and responsive primary cells. Proc. Natl. Acad. Sci. USA **84**:3802–3806.
12. **Koski, I. R., D. G. Poplack, and R. M. Blaese.** 1976. A nonspecific esterase stain for the identification of monocytes and macrophages, p. 359–362. *In* B. R. Bloom and J. R. David (ed.), *In vitro* methods in cell-mediated and tumor immunity. Academic Press, Inc., New York.
13. **Kurt-Jones, E. A., D. I. Beller, S. B. Mizel, and E. R. Unanue.** 1985. Identification of a membrane-associated interleukin 1 in macrophages. Proc. Natl. Acad. Sci. USA **82**:1204–1208.
14. **Lane, F. C., and E. R. Unanue.** 1972. Requirement of thymus (T) lymphocytes for resistance to listeriosis. J. Exp. Med. **135**: 1104–1112.
15. **Larrick, J. W., and S. L. Kunkel.** 1988. The role of tumor necrosis factor and interleukin 1 in the immunoinflammatory response. Pharm. Res. **5**:129–139.
16. **Lee, S.-H., P. R. Crocker, and S. Gordon.** 1986. Macrophage plasma membrane and secretory properties in murine malaria. Effects of *Plasmodium yoelii* infection of macrophages in the liver, spleen and blood. J. Exp. Med. **163**:54–74.
17. **Lepay, D. A., C. F. Nathan, R. M. Steinman, H. W. Murray, and Z. A. Cohn.** 1985. Murine Kupffer cells. Mononuclear phagocytes deficient in the generation of reactive oxygen intermediates. J. Exp. Med. **161**:1079–1096.
18. **Lepay, D. A., R. M. Steinman, C. F. Nathan, H. W. Murray, and Z. A. Cohn.** 1985. Liver macrophages in murine listeriosis. Cell-mediated immunity is correlated with an influx of macrophages capable of generating reactive oxygen intermediates. J. Exp. Med. **161**:1503–1512.
19. **Mackaness, G. B.** 1962. Cellular resistance to infection. J. Exp. Med. **116**:381–406.
20. **Magee, D. M., and E. J. Wing.** 1988. Cloned L3T4$^+$ T lymphocytes protect mice against *Listeria monocytogenes* by secreting IFN-γ. J. Immunol. **141**:3203–3207.
21. **Magee, D. M., and E. J. Wing.** 1988. Antigen-specific production of colony-stimulating factors by *Listeria monocytogenes*-immune L3T4 positive cells. J. Infect. Dis. **157**:941–948.
22. **Magee, D. M., and E. J. Wing.** 1989. Secretion of colony-stimulating factors by T cell clones: role in adoptive protection against *Listeria monocytogenes*. J. Immunol. **143**:2336–2341.
23. **Magilavy, D. B., and J. L. Rothstein.** 1988. Spontaneous production of tumor necrosis factor-α by Kupffer cells of MRL/lpr mice. J. Exp. Med. **168**:789–794.
24. **Meltzer, M. S.** 1981. Use of peroxidase stain by the Kaplow method, p. 363–366. *In* D. O. Adams, P. J. Edelson, and H. Koren (ed.), Methods for studying mononuclear phagocytes. Academic Press, Inc., New York.
25. **Mosmann, T.** 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunol. Methods **65**:55–63.
26. **North, R. J.** 1970. The relative importance of blood monocytes and fixed macrophages to the expression of cell-mediated immunity to infection. J. Exp. Med. **132**:521–534.
27. **North, R. J.** 1973. The mediators of anti-*Listeria* immunity as an enlarged population of short-lived, replicating T-cells: kinetics of their replication. J. Exp. Med. **138**:342–355.
28. **Richman, L. K., R. J. Klingenstein, J. A. Richman, W. Strober, and J. A. Berzofsky.** 1979. The murine Kupffer cell. I. Characterization of the cell serving accessory function in antigen-specific T cell proliferation. J. Immunol. **123**:2602–2609.
29. **Rosen, H., S. Gordon, and R. J. North.** 1989. Exacerbation of murine listeriosis by a monoclonal antibody specific for the type 3 complement receptor of myelomonocytic cells. Absence of monocytes at infective foci allows *Listeria* to multiply in non-phagocytic cells. J. Exp. Med. **170**:27–37.
30. **Weaver, C. T., C. M. Hawrylowicz, and E. R. Unanue.** 1988. T helper cell subsets require the expression of distinct costimulatory signals by antigen-presenting cells. Proc. Natl. Acad. Sci. USA **85**:8181–8185.
31. **Wing, E. J., A. Waheed, and R. K. Shadduck.** 1984. Changes in serum colony-stimulating factor and monocytic progenitor cells during *Listeria monocytogenes* infection in mice. Infect. Immun. **45**:180–184.
32. **Wong, G. G., and S. C. Clark.** 1988. Multiple actions of interleukin 6 within a cytokine network. Immunol. Today **9**: 137–139.