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In Vitro Propagation of Antigen-Specific T Lymphocytes that Adoptively Transfer Resistance to *Listeria monocytogenes*

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Murine T cells generated against heat-killed *Listeria monocytogenes* or *Listeria* intracellular product (LIP) were propagated in a source of Interleukin 2. Both T-cell cultures were greater than 98% Lyt 1^+, $2/3^-$ and proliferated specificially against LIP and *L. monocytogenes* crude whole-cell antigen in vitro. Proliferation of both T-cell cultures required the presence of antigen and accessory cells syngeneic to the T cells at the left end of the major histocompatibility complex. The ability of these cultures to adoptively transfer protection against challenge with viable *Listeria* cells was dramatically different. As few as 10^6 LIP-specific T cells conferred significant protection against a lethal challenge of *Listeria* cells, whereas cultures induced against crude whole-cell antigen showed little or no protective function. The resistance conferred by LIP-specific T cells was specific in that the cells did not reduce the mortality seen after challenge with *Salmonella typhimurium*.

Resistance in mice to infection with *Listeria monocytogenes*, a facultative intracellular parasite, involves collaboration between immune T lymphocytes and activated macrophages (16–18). Thymus-derived lymphocytes appear to mediate acquired resistance by releasing soluble factors (lymphokines) after antigenic stimulation which in turn increase nonspecific bactericidal activity of macrophages (13, 22, 27). Historically, the adoptive transfer to naive recipients of resistance against *Listeria* cells has been achieved only with large numbers of spleen cells derived from actively immunized hosts (2, 14, 18, 29) and not after infusion of hyperimmune serum (20). Recent studies, however, indicate that T-cell lines generated against viable *Listeria* cells provide protection (10).

The inherent low frequency of antigen-specific T lymphocytes present in an intact spleen or lymph node obtained from an immune animal makes it difficult to analyze effectively the interactions of various cell types during the development of an immune response. To effectively study the network of communication that exists among various cell types important in the development of an immune response, it is necessary to develop a system that allows for the culture and long-term survival of antigen-specific T cells and which ultimately results in an increased frequency of antigen-reactive cells in the absence of antigen. Numerous recent reports indicate that T lymphocytes can be propagated in sources of Interleukin 2 (IL-2) present in lectin-induced normal spleen cells and that these propagated cells retain their antigenic specificity as well as functional activity (8, 21, 25, 26).

In the experiments presented here, a water-soluble antigenic preparation, referred to as *Listeria* intracellular product (LIP), was used to generate antigen-specific T lymphocytes in vitro from lymph node cells (LNC) obtained from LIP-primed animals. These antigen-primed LNC were used to evaluate, first, whether the low frequency of antigen-reactive T lymphocytes present in lymph nodes of LIP-primed animals could be increased by propagation in IL-2, and second, whether these IL-2-propagated T lymphocytes retained antigenic specificity as well as functional activity; i.e., were they effective in adoptively transferring resistance to naive recipients?

MATERIALS AND METHODS

Mice. Eight- to 10-week-old male (C57BL/6 × DBA/2)F_1 (BDF_1) and A/J mice were produced and maintained in the animal care facilities at National Jewish Hospital and Research Center, Denver, Colo. Animals were maintained on Lab Blox (Wayne, Allied Mills, Chicago, Ill.) and hyperchlorinated water ad libitum.

Mitogens and antigens. Ovalbumin (OVA) was purchased from Sigma Chemical Co. (St. Louis, Mo.).

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Concanavalin A (ConA) was purchased from Pharmacia Fine Chemicals, (Piscataway, N.J.). Lipopolysaccharide was purchased from Difco Laboratories (Detroit, Mich.). *Listeria* intracellular product (LIP) is a water-soluble supernatant material obtained from log-phase cultures of *L. monocytogenes* (11). Briefly, washed *L. monocytogenes* cells, serotype 1, strain 10403, were mechanically disrupted, using a French press at 20,000 lb/in^2. The supernatant material was separated from cell wall fragments by high-speed centrifugation at 105,000 × *g* for 2 h, filtered through a 0.22-μm filter (Millipore Corp., Bedford, Mass.), and stored at −70°C. A crude whole-cell antigen derived from *L. monocytogenes*, referred to as LM, was prepared from an overnight culture of *Listeria* cells that were washed three times in sterile distilled water, suspended in sterile saline, and inactivated by autoclaving.

Bacterial cultures and maintenance. *L. monocytogenes*, serotype 1, strain 10403, and *Salmonella typhimurium* (ATCC 14028) were maintained in a virulent state by repeated passages in mice. *L. monocytogenes* had an intravenous 50% lethal dose (LD_{50}) of approximately 1.5 × 10^4 organisms. *S. typhimurium* had an intravenous LD_{50} of approximately 2.3 × 10^4 organisms. The bacteria were stored frozen at −70°C suspended in brain heart infusion broth (Difco Laboratories). The cultures were periodically checked for purity. Suspensions for immunization or challenge were prepared from log-phase cultures which were diluted in 1% peptone water to contain the desired number of bacteria in 0.1 ml of inoculum.

Preparation of ConA supernatant containing IL-2. Single-cell suspensions were prepared by gently teasing apart individual spleens of inbred Lewis rats into cells which were washed, counted, and suspended in RPMI 1640 medium (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Calbiochem, La Jolla, Calif.), 1% gentamicin (Sigma Chemical Co.), 2 mM L-glutamine (GIBCO Laboratories), 5% fetal calf serum, and 5 × 10^{-5} M 2-mercaptoethanol. Spleen cell suspensions containing 3 × 10^6 cells per ml were cultured with 2.5 μg of ConA per ml and incubated for 24 h under 5% CO at 37°C. At the end of incubation period, all cell suspensions were centrifuged at 100 × *g* for 10 min, and supernatant material was harvested and filter sterilized. Before use, this supernatant fluid was diluted to 50% with fresh medium and supplemented with alpha-methyl mannoside at a final concentration of 20 mg/ml to neutralize the mitogenic activity of ConA.

Generation of antigen-specific T-cell cultures. Antigen-specific T-cell cultures obtained by the methods to be described below will henceforth be referred to as T-cell lines. Draining inguinal and periaortic lymph nodes were removed from mice primed 7 days previously with 100 μg of LIP, OVA, or lyophilized heat-killed LM emulsified in complete Freund adjuvant. All immunizations were administered subcutaneously at the base of the tail (4). Lymph nodes were teased into single-cell suspensions in ice-cold RPMI 1640 medium. Washed single cells were suspended at 4 × 10^6 cells per ml in Click medium supplemented with 1% normal mouse serum, 5 × 10^{-5} M 2-mercaptoethanol, 1% gentamicin, and 10 μg of homologous antigen per ml and seeded in 24-well cluster plates (Costar, Cambridge, Mass.) at 1 ml per well. After 4 days, lymphoblasts were enriched on Ficoll-Isopaque gradients with a density of 1.09 (5), washed twice, seeded in supplemented Click media with rat ConA supernatants containing 10 U of IL-2 per ml, and grown for 47 days. ConA-induced culture supernatants were monitored for IL-2 activity, using a standard microassay based upon the IL-2-dependent proliferation of a mouse cytotoxic T-cell line (8). These rapidly dividing T cells have been maintained for more than 3 months by antigenic stimulation every 2 weeks in fresh medium with homologous antigen (10 μg/ml) and gamma-irradiated (3,000 R from a ^{137}Cs source) syngeneic spleen cells in the presence of IL-2. The ratio of T-cell blasts to irradiated spleen cells was adjusted at these times to 1:10.

Lymphocyte proliferation assay. T-cell lines from LIP-, LM-, and OVA-primed cultures were tested for their ability to proliferate against antigens in the presence of accessory cells from normal mice as previously described (26). Briefly, 5 days before the assay, T cells were seeded at 8 × 10^4 cells per ml in Click supplemented media with 10 U of IL-2 per ml only. On day zero, they were harvested, washed, and seeded at 10^4 cells per microtiter well in supplemented Click medium. To some groups we added gamma-irradiated spleen cells (3,000 R) at 4 × 10^5 cells per ml. Triplicate wells were stimulated with LIP, LM, OVA (all at 100 μg/ml), or ConA (1 μg/ml) and incubated for 2 days at 37°C. Proliferation was determined by the incorporation of 1 μCi of [^{3}H]thymidine (New England Nuclear Corp., Boston, Mass.) per well for an additional 18 h. All wells were harvested with a Titertek cell harvester (Flow Laboratories, McLean, Va.) and counted by liquid scintillation.

Cytofluorography. T-cell lines from LIP-, LM-, and OVA-primed cultures grown in a source of IL-2 for 7 days before testing were evaluated for the presence of Thy-1 antigen and Lyt 1 and 2 surface markers. Cultures with greater than 95% viability were incubated with anti-Thy 1.2, anti-Lyt 1, or anti-Lyt 2 monoclonal antibodies (Becton, Dickinson & Co., Sunnyvale, Calif.) for 60 min on ice in the presence of 2% normal goat serum. After washing, a 1/20 dilution of fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin (Bionetic, Kensington, Md.) was added for 30 min on ice. After dilution to 2 ml in saline, all cell suspensions were analyzed with an Ortho Diagnostics System Cytofluorograf model 50 HH with a system 2150 computer by exciting with light (0.5 W) with a wavelength of 488 nm, using a Lexel laser.

Cell transfer. BDF_1 mice received an intravenous injection of various numbers of LIP-, LM-, or OVA-primed T-lymphocyte cell lines or 10^6 LIP-primed LNC. Approximately 3 h later, each mouse received 10 LD_{50} of either *L. monocytogenes* or *S. typhimurium*. Mice in each group were monitored daily for 1 week, and the total number of survivors in each group was determined. Mice that survived beyond the day 7 recovered completely from the infection.

RESULTS

Antigen-induced proliferation of LIP- and LM-specific T-cell lines. We evaluated the antigen specificity of our T-cell lines by antigen-induced proliferation, shown to be specific in murine

systems for Lyt 1^+ T cells for soluble protein antigens and protozoan antigens after 4 and 30 days of culture (15, 26). To determine if LIP-responsive T cells were enriched during in vitro propagation, we compared the antigen-induced stimulation of equal numbers of LIP-primed LNC with IL-2-propagated T cells. Data presented in Fig. 1 indicate that LIP-stimulated IL-2-propagated T cells in the presence of irradiated syngeneic accessory cells showed a significant response to LIP which was dependent on the number of T cells in the culture. Significant proliferation was observed with as few as 2.5×10^3 T cells per well. In contrast, LIP-primed LNC responded to LIP to a lesser extent and, even at 20,000 cells per well, showed a maximal stimulation index of 22 compared with a maximal stimulation index of 136 with the IL-2-grown LIP-specific T-cell line. Both LNC and the LIP-stimulated T-cell line responded comparably to the T-cell mitogen ConA, indicating that the differential proliferation seen with LIP was not due merely to fewer T cells present in the LNC population. These data suggest that there is a progressive enrichment of LIP-reactive cells during in vitro stimulation and propagation of LIP-primed LNC. These data also indicate the requirement for irradiated accessory cells for antigen-induced stimulation, since no proliferation was observed at any T-cell concentration in the absence of accessory cells. Neither the LNC nor the LIP-induced T-cell line responded to an unrelated antigen (OVA) at any antigen concentration or T-cell density, indicating the specificity of the response.

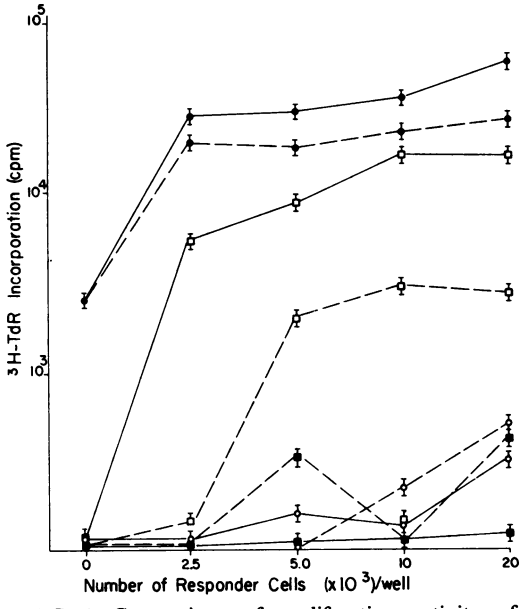

FIG. 1. Comparison of proliferative activity of BDF_1 cells from LIP-primed lymph nodes (dashed lines) and these same cells after stimulation in vitro with LIP and expansion in IL-2 (solid lines). Various numbers of these responder cells were placed into wells containing irradiated syngeneic accessory cells and stimulated for 3 days with ConA (1 μg/ml) (●), LIP (100 μg/ml) (□), OVA (100 μg/ml) (■), or saline (○). The cultures were pulsed with [^{3}H]thymidine (^{3}H-TdR) (1 μCi per well) for the last 18 h of culture, and the cells were harvested. The T cells from the IL-2 cultures did not respond to mitogen or antigen in the absence of fillers (<150 cpm). The LIP-primed LNC alone responded minimally to LIP (760 cpm). BDF_1 T-cell blasts primed against OVA and propagated in IL-2 were stimulated by OVA (100 μg/ml) in the presence of syngeneic accessory cells in a simultaneous experiment (15,794 ± 1,486 cpm). All points represent the mean counts per minute ± standard error of the mean of triplicate cultures.

We then compared the proliferative response of the LIP-specific T-cell line with a continuous T-cell line obtained from LNC primed in vivo with heat-killed *Listeria* cells and restimulated in vitro with LM. Data presented in Fig. 2 illustrate that LIP-specific T cells responded better to LM than to LIP at higher concentrations of T cells. T cells primed with LM showed a small but significantly better response to LM than to LIP. However, it is clear that LIP-reactive T cells were generated during in vivo or in vitro stimulation, or both, with whole *Listeria* cells, presumably since LM contains significant amounts of this LIP antigen(s). This becomes an important point when the functional activity of these two lines in vivo is evaluated below.

Genetic restriction of proliferative response. Several investigators have shown that the interaction between *Listeria*-immune T cells and macrophages is major histocompatibility complex-restricted and that this restriction maps to the I region of the major histocompatibility complex (7, 29). We wanted to determine if antigen-induced proliferation of LIP- and LM-specific T-cell lines would show similiar major histocompatibility complex restriction. Figure 3 shows the results of an experiment in which BDF_1 T-cell lines reactive with LIP or LM were tested on irradiated accessory cells allogeneic or syngeneic to T cells at the K end of the major histocompatibility complex. As shown, LIP- and LM-specific T cells responded well to LIP and LM on syngeneic (BDF_1) accessory cells. Background levels of proliferation of both T-cell lines were seen on semiallogeneic accessory cells obtained from A/J mice. The ConA response of both lines was significant on both BDF_1 and A/J accessory cells and, therefore, genetically unrestricted, as has been reported by others (26). This also indicates that the A/J fillers produced no suppressive substances which inhibited antigen-induced proliferation. As was seen before,

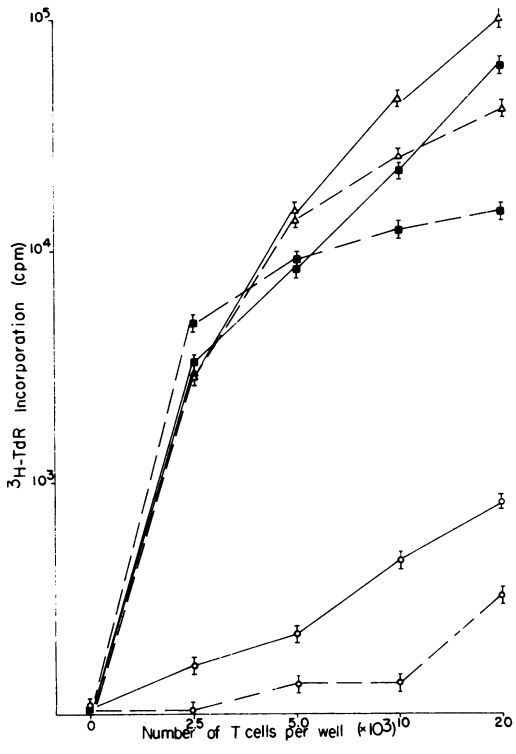

FIG. 2. Comparison of proliferative responses of LIP-primed T cells (dashed lines) and LM-primed T cells (solid lines) to soluble LIP (100 μg/ml) (■) and to LM (100 μg/ml) (△). The antigen control was saline (○). Various numbers of responder IL-2-grown T cells were placed into wells containing 4×10^5 irradiated syngeneic BDF_1 splenic accessory cells with or without antigen for 3 days. The cultures were pulsed with [^{3}H]thymidine (^{3}H-TdR) (1 μCi per well) for the last 18 h, and the cells were harvested and counted. The responses of the LIP T cells and the LM T cells to the unrelated antigen OVA (100 μg/ml) were 321 and 887 cpm, respectively, at 2×10^4 T cells per well with irradiated syngeneic accessory cells. Neither LIP-primed T cells or LM-primed T cells responded to soluble LIP or LM at any concentration in the absence of irradiated accessory cells (<150 cpm). All points represent the mean counts per minute ± standard error of the mean of triplicate cultures.

neither T cell line responded to the unrelated antigen OVA or to the B-cell mitogen, lipopolysaccharide.

Surface phenotype of T-cell lines. T cells specific for LIP, LM, and OVA were tested for surface phenotype only after 7 days of culture in IL-2 as described above. These cells were greater than 98% positive for the Thy 1.2 and Lyt 1 surface antigens. In addition, each of these cell lines contained undetectable numbers of Lyt 2/3-positive cells.

Specific protection against listeriosis after adoptive transfer of LIP-specific T-cell line. The in vivo functional activity of LIP- and LM-induced continuous T-cell lines was evaluated 4 to 7 days after propagation in IL-2-containing supernatant material by transferring various numbers of T cells intravenously into naive syngeneic recipients followed 3 h later with a challenge dose of 10 LD_{50}'s of viable *Listeria* cells. To determine the specificity of protection, two approaches were followed. First, an OVA-specific T-cell line generated identically as LIP T-cells was injected intravenously, followed by challenge with viable *Listeria* cells into naive recipients. Second, animals which received LIP T cells were challenged with 10 LD_{50} of *S. typhimurium*.

The cumulative results of five experiments are presented in Table 1 and illustrate that greater than 90% of the mice receiving 10^6 LIP T cells survived a challenge with 10 LD_{50}'s of *Listeria* cells. The percent protection was dependent on the number of LIP-reactive T cells transferred. In contrast, those animals which received either 10^6 LIP-primed LNC or T cells primed to an unrelated antigen (OVA) all died. In addition, the T-cell line primed with LM failed to provide protection against 10 LD_{50}'s of *Listeria* cells except at 10^6 T cells per mouse, an amount which provided a survival rate of only 4%. The same results were observed when LIP- and LM-induced T-cell lines were generated de novo on five separate occasions. Interestingly, this LM-primed T-cell line proliferated in vitro to both LIP and LM yet had no detectable protective effect under conditions in which LIP T cells were especially efficacious. Finally, LIP T cells did not protect against a challenge of viable *S. typhimurium*, a facultative intracellular organism antigenically unrelated to *L. monocytogenes*. It was also observed that mice receiving 10^6 LIP T cells and 10 LD_{50}'s survived longer than 2 months and were resistant to rechallenge with 100 LD_{50}'s of *Listeria* cells at that time.

DISCUSSION

In vitro long-term culture of antigen-specific T lymphocytes has provided a means by which to study the complex interactions that occur between various cell types and that ultimately lead to the development of an immune response. It has been shown, for example, that cytolytic or helper T lymphocytes can be maintained in vitro for long periods of time and that these propagated cells retain both the property of antigen specificity and the property of genetic restriction in vitro (3, 8, 9, 15, 25, 26). Tees and Schreier (28) demonstrated the ability to reconstitute athymic nude mice with cloned specific helper T cells, resulting in a plaque-forming response comparable to that observed in normal mice.

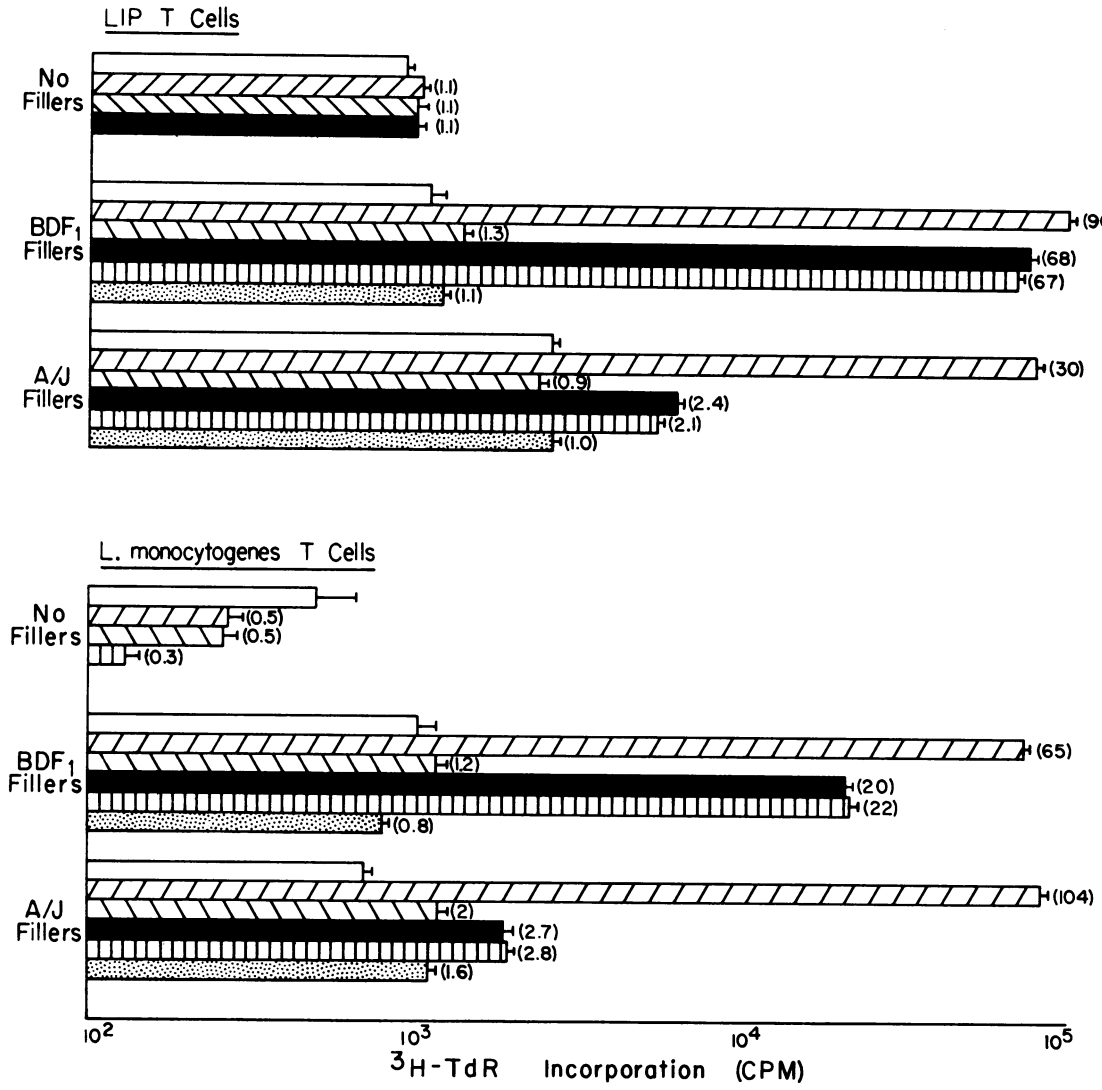

FIG. 3. Proliferative response of IL-2-grown T cells from mice primed with LIP (LIP T cells) or LM (LM T cells) in vivo. Each group of BDF_1 mice was primed with LIP or LM, respectively, and the T cells from the lymph nodes were expanded in IL-2 as described in the text. Each group of T cells was seeded into microtiter wells (2×10^4 cells per well) and stimulated with saline (□), ConA (1 μg/ml) (▨), LPS (1 μg/ml) (▧), LIP (100 μg/ml), (■), LM (100 μg/ml) (▩), or OVA (100 μg/ml) (▦), in either the presence or the absence of irradiated accessory cells (4×10^5 cells per well). The accessory cells were either syngeneic (BDF_1) or allogeneic (A/J) with the T cells. Irradiated accessory cells alone did not respond either to LIP or LM (<800 cpm). Values are expressed as mean counts per minute ± standard error of the mean of triplicate cultures. Numbers in parentheses represent the stimulation index.

Recently, Barry and Hinrichs (1) found that a 3-day incubation of *Listeria*-immune spleen cells with ConA before adoptive transfer increased the antilisterial activity of *Listeria*-immunized mice. In addition, Kaufman and Hahn (10) reported that the long-term culture of T-cell lines derived from mice infected with live *Listeria* cells provided T cells that were capable of adoptively transferring delayed-type hypersensitivity and protection to naive recipients against infection with *L. monocytogenes*.

This report presents data demonstrating that a water-soluble extract, LIP, derived from mechanically disrupted *L. monocytogenes* primed T cells in vivo, cells which subsequently were grown in the presence and then absence of antigen in vitro. These LIP-induced T lymphocytes were antigen specific (Fig. 1 and 2) and, in

addition, could transfer specific resistance to infection with *Listeria* cells (Table 1). Equivalent low numbers of LIP-primed LNC, before in vitro propagation, failed to mount a detectable in vitro proliferative response and were also unable to transfer resistance to *Listeria* cells. These experiments suggest that antigen-induced cell proliferation results in the enrichment of an LIP-reactive and *Listeria*-protective T-cell population even after a relatively short period of time in culture. T cells generated against an unrelated antigen (OVA) or against a crude cell extract derived from whole *Listeria* cells (LM) did not confer protection against viable *Listeria* cells, indicating that the protection observed with the LIP T-cell line was not merely a property of in vitro-propagated T cells. In addition, the LIP-reactive T cells were not capable of non-specifically activating macrophages in vivo to protect against a live *Salmonella* challenge in the absence of LIP.

Although the mechanism responsible for induction of resistance by these few cells is not clear, it seems likely that the selection procedure enriches for a population of T cells which have a specialized function, e.g., lymphokine production. We have data which identifies these LIP-reactive T cells as Lyt 1^+, $2/3^-$, suggesting a possible role for these LIP-reactive T cells as amplifier or helper T cells which activate macrophages. In addition, supernatant material obtained from these cultured LIP-reactive T cells, when assayed in the indirect macrophage migration inhibition assay, showed greater than 65% inhibition of migration (data not shown).

These LIP-reactive T cells may be especially efficacious against listeriosis because, fortuitously, this crude extract may contain unique antigens important for the generation of the T cells which mediate protection. Other experiments suggest that microgram quantities of LIP, when injected intravenously, are capable of protecting mice against a lethal challenge of *L. monocytogenes* (unpublished observation). This is interesting since other components derived from *Listeria* cells either provide marginal protection or require extensive immunization to induce protection in normal animals (6, 12, 19, 23, 24). The fact that T cells generated against whole, killed *Listeria* cells (LM) were ineffective in providing any protection supports the concept that there may be uniquely important antigens associated with the LIP extract. The inability of LM-reactive T lymphocytes to provide protection (Table 1) suggests one of the following. First, these IL-2-propagated LM T cells may not express the appropriate function in the recipient animal, e.g., lymphokine production. Second, the majority of these LM T lymphocytes may be primed to an antigen(s) which is irrelevant in providing protection. Last, the majority of these LM-reactive cells may function as suppressor T lymphocytes or inducers of suppressor cells in vivo. However, phenotypic characterization of these cells indicate that they are not classical Lyt 1^-, $2/3^+$ T cells. Studies to distinguish between these possibilities are in progress and should provide information on the induction of immunity to an important intracellular parasite.

This manuscript presents evidence which demonstrates that T cells primed to a subcellular bacterial antigen can be grown in vitro and can

TABLE 1. Adoptive transfer of resistance to *L. monocytogenes* by antigen-stimulated T lymphocytes grown in vitro

Cells transferred[a]	Challenge organism[b]	No. of survivors/ total challenged[c]
None	*L. monocytogenes*	0/25 (0)
LIP IL-2-propagated T lymphocytes	*L. monocytogenes*	
1×10^5		3/15 (20)
5×10^5		12/15 (80)
1×10^6		23/25 (92)
LM IL-2-propagated T lymphocytes	*L. monocytogenes*	
1×10^5		0/15 (0)
5×10^5		0/15 (0)
1×10^6		1/25 (4)
OVA IL-2-propagated T lymphocytes	*L. monocytogenes*	
1×10^5		0/15 (0)
5×10^5		0/15 (0)
1×10^6		0/25 (0)
LIP LNC	*L. monocytogenes*	
1×10^5		ND[d]
5×10^5		ND
1×10^6		0/25 (0)
None	*S. typhimurium*	0/25 (0)
LIP IL-2-propagated T lymphocytes (1×10^6)	*S. typhimurium*	0/25 (0)

[a] BDF_1 mice received an intravenous injection of either LIP-, LM-, or OVA-primed T lymphocytes that were propagated for 96 h in ConA supernatant fluid containing IL-2. A fourth group of animals received an intravenous injection of LIP-primed LNC. Results represent the composite of five separate experiments.

[b] Mice in each group were challenged intravenously with 10 LD_{50} of either *L. monocytogenes* or *S. typhimurium* 3 h after cell transfer.

[c] Number in parentheses represents percentage of survivors.

[d] ND, Not done.

transfer protection against a lethal challenge of the homologous bacteria. These findings indicate that these T cells are highly enriched for the effector function(s) which appears to be crucial in resistance to intracellular bacteria. In addition, this relatively homogeneous population of effector T cells may be used to study cellular interactions operative in the immune response to important pathogens.

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