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Use of Recombinant Interleukin-2 To Enhance Adoptive Transfer of Resistance to *Listeria monocytogenes* Infection

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In vitro incubation of *Listeria*-immune spleen cells (LISC) with recombinant interleukin-2 (rIL-2) for at least 3 days increased their ability to transfer antilisteria resistance to recipient mice. This effect was blocked by the in vitro addition of transforming growth factor β_1. The level of protection afforded by the transfer of rIL-2-incubated LISC was further elevated by the in vivo administration of rIL-2 at a dose that by itself did not significantly increase antilisteria resistance. The antilisteria resistance of recipient mice remained elevated for approximately 7 days and then rapidly declined to undetectable levels by 10 days. After cell transfer, recipient mice were protected against challenge with *Listeria monocytogenes* but not *Salmonella typhimurium*, *Yersinia enterocolitica*, or *Streptococcus pyogenes*. Flow cytometric analyses revealed an increase in the percentages of $CD8^+$, NK^+, and $\gamma\delta$ T cell receptor$^+$ cells but no change in the percentage of $CD4^+$ cells as a result of LISC coculturing with rIL-2. In vitro depletion of $CD4^+$ cells just prior to transfer had no significant effect on the adoptive transfer of resistance; depletion of $CD8^+$ cells reduced the level of resistance by approximately 25%. Combined depletion of Thy-1.2^+, $CD4^+$, and $CD8^+$ cells just prior to adoptive transfer diminished the level of protection in the spleens but not the livers of recipient mice. These data suggest that rIL-2 can be used to augment adoptive immunotherapy for bacterial infection in a manner similar to adoptive immunotherapy of human cancer patients. Although the protective cell population was not definitively identified, it appeared to be independent of $CD4^+$ cells and only partly dependent on $CD8^+$ cells.

Listeria monocytogenes is a facultative intracellular pathogen that evokes a strong T cell-mediated immune response in infected animals (29, 32, 49). As a result, it has been extensively used as a model for studying the regulation of cellular immunity to bacterial infection (50, 51). Early studies demonstrated that antigen-dependent lymphoid cells can adoptively transfer antilisteria resistance to naive animals (29, 35). It was subsequently shown that $CD8^+$ T cells in suspensions of freshly isolated *Listeria*-immune spleen cells (LISC) are the predominant cell type responsible for the adoptive transfer of antilisteria resistance (8, 25, 31). Although a high level of immunity (i.e., a 2- to 3-$\log_{10}$ reduction in peak bacterial burden) can be transferred by this method, it requires large numbers (5×10^7 to 1×10^8) of LISC (6, 8, 25, 31). More recent studies with *L. monocytogenes*-specific T cell lines and T cell clones have provided evidence that both $CD8^+$ and $CD4^+$ T cells contribute to antilisteria resistance (6, 24, 25, 31, 45).

Several studies have shown that antimicrobial resistance can be potentiated by recombinant interleukin-2 (rIL-2), a cytokine that is pivotal in T cell activation and proliferation (47). We reported previously that the exogenous administration of human rIL-2 protected mice against *L. monocytogenes* infection (18). Other investigators have demonstrated that rIL-2 enhances resistance to various pathogenic microbes (5, 10, 21, 23). In this study, we explored the possibility that rIL-2 could be used to augment the adoptive transfer of antilisteria resistance by LISC. Our data indicate that preincubation with rIL-2 allows relatively small numbers of LISC (10^6) to transfer antilisteria resistance by a mechanism that appears to be largely independent of $CD4^+$ cells and only partly dependent on $CD8^+$ cells.

MATERIALS AND METHODS

Mice. Male $(C57BL/6 \times DBA/2)F_1$ mice, stated by the supplier to be free of infection by adventitious agents such as Sendai virus and mouse hepatitis virus, were obtained from Jackson Laboratory (Bar Harbor, Maine) at 5 to 6 weeks of age. The mice were housed under plastic microisolator caps (Lab Products, Frederick, Md.) at the School of Veterinary Medicine, University of Wisconsin, Madison, and given Purina Lab Chow (Ralston Purina, St. Louis, Mo.) and water ad libitum. Mice were allowed to acclimate to our animal care facility for at least 1 week prior to use in experiments.

rIL-2. Human rIL-2 (lots 9P87, 1049, and 2059) was kindly provided by M. Gately (Hoffmann-La Roche Inc., Nutley, N.J.). The rIL-2 was expressed in *Escherichia coli* and contained less than 5 U (less than 1 ng) of endotoxin per mg, as determined by the *Limulus* amebocyte lysate assay. The specific activity of all lots was approximately 1.5×10^7 U/mg, as assessed by the CTLL-2 assay. Appropriate working dilutions were made with pyrogen-free saline adjusted to pH 7.4.

Preparation of LISC. Mice were immunized intravenously (i.v.) with 5×10^3 *L. monocytogenes* cells in 0.2 ml of pyrogen-free saline. Six or 7 days later, the mice were killed by cervical dislocation. The spleens were removed and passed through a wire screen to disperse the cells, and the cells were washed by centrifugation. LISC were either transferred immediately to recipient mice or incubated in vitro prior to transfer. Untreated LISC were resuspended in Hank's balanced salt solution (HBSS) (GIBCO Laboratories, Grand Island, N.Y.) at 2×10^6 cells per ml and injected i.v. into a lateral tail vein. When the LISC were to be

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preincubated in vitro before transfer, they were resuspended at 2×10^6 cells per ml in RPMI 1640 (GIBCO) containing 0.5 μg of rIL-2 per 10^6 LISC, 10% fetal bovine serum (GIBCO), and 25 μg of gentamicin (GIBCO) per ml, and the suspension was incubated for 3 days at 37°C with 5% CO_2 (unless otherwise indicated) in 25-cm^2 tissue culture flasks (Falcon; Becton-Dickinson Labware, Oxnard, Calif.). Then, the nonadherent LISC were removed from the flasks, washed twice with HBSS, and resuspended in HBSS at 2×10^6 cells per ml. Naive recipient mice were injected i.v. with 0.5 ml of the cell suspension via a lateral tail vein. In some experiments, purified human platelet-derived transforming growth factor β_1 ($TGF\beta_1$) was added to the LISC cultures at concentrations shown previously to inhibit spleen cell proliferation in vitro (unpublished data). $TGF\beta_1$ was acid activated in accordance with the supplier's directions (R & D Systems, Inc., Minneapolis, Minn.).

MAbs. We obtained from the American Type Culture Collection (Rockville, Md.) hybridoma cell lines secreting the following monoclonal antibodies (MAbs): anti–Thy-1.2 (30-H12; rat immunoglobulin G2b [IgG2b]), a pan-T cell marker MAb; anti–Lyt-2/CD-8 (53-6.72; rat IgG2a), recognizing $CD8^+$ T cells; anti–L3T4/CD-4 (GK1.5; rat IgG2b), specific for $CD4^+$ T cells; anti–NK-1.1 (PK 136; mouse IgG2b), directed against natural killer (NK) cells; anti-B220/CD45R (RA3-6B2; rat IgG2a), which reacts with pre-B and B lymphocytes as well as lymphocytes with cytotoxic activity; and anti-Ia^k (11.5.2.1.9; mouse IgG2b), an irrelevant MAb. A hybridoma cell line secreting a hamster IgG directed against mouse γδ T cells (GL3.1A) was generously provided by L. LeFrancois of The Upjohn Pharmaceutical Co. (Kalamazoo, Mich.). All hybridoma cell lines were grown as ascites in pristane-primed flora-defined *nu/nu* BALB/c mice (University of Wisconsin—Madison Gnotobiotic Laboratory, Madison). MAbs were purified by application of clarified ascitic fluid to a DEAE-5PW high-pressure liquid chromatography column (Beckman Instruments, Inc., Fullerton, Calif.) and elution with a linear gradient of 50 to 700 nM sodium acetate in 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid buffer (Sigma Chemical Co., St. Louis, Mo.). For verification of antibody purification, samples of purified antibodies were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis by the method of Laemmli (28) and stained with Coomassie blue to visualize the proteins. The protein content of the purified antibodies was determined by use of the bicinchoninic acid technique as described by the supplier (Pierce Chemical Co., Rockford, Ill.). Purified MAbs were divided into aliquots and stored at −70°C.

For immunofluorescence staining, fluorescein-conjugated and phycoerythrin-conjugated forms of the above-mentioned MAbs were purchased from Pharmingen (San Diego, Calif.).

T cell subset depletion. After the LISC were incubated with rIL-2 for 72 h, the nonadherent cells were removed, washed by centrifugation, and resuspended in RPMI 1640 with 10% fetal bovine serum at a cell density of 10^7 cells per ml. The LISC were incubated in an ice bath for 1 h with 5 μg of the appropriate MAb per 10^6 LISC. The cells were washed, resuspended in an equal volume of sterile rabbit complement (Lo-Tox M; Accurate Chemical, Westbury, N.Y.), and incubated in a 37°C water bath for 1 h. After being washed, the cells were applied to a Lympholyte density gradient (Accurate Chemical) to remove the dead cells, washed again, and resuspended in HBSS at 2×10^6 cells per ml for adoptive transfer. The resulting cell suspensions were routinely >98% viable, as determined by trypan blue exclusion.

Fluorescence-activated cell sorter analysis. The expression of cell surface markers by spleen cells was assessed by fluorescence-activated cell sorter analysis with fluorescein-conjugated or phycoerythrin-conjugated MAbs (Pharmingen). Portions of the LISC suspensions were removed, washed in HBSS containing 0.1% sodium azide, and incubated with the appropriate MAb (4 μg per 10^6 cells) for 40 min at 4°C. The cells were washed once by centrifugation and then resuspended in 0.8 ml of HBSS with 0.1% azide. Stained cells were analyzed immediately with an Epics-C flow cytometer and the IMMUNO program (Coulter Electronics, Hialeah, Fla.).

Bacterial infection. *L. monocytogenes* EGD was maintained as described previously (18). Log-phase listeriae were suspended in broth containing 20% glycerol, and aliquots were stored at −70°C. For each experiment, the listeriae were freshly thawed and diluted in pyrogen-free saline at appropriate working concentrations immediately prior to injection. Mice were infected i.v. via a lateral tail vein with 5×10^4 viable listeriae in a total volume of 0.2 ml. Three days later, the mice were killed by cervical dislocation and their spleens and livers were removed to separate sterile tissue grinders containing 5 ml of phosphate-buffered saline (PBS). Each organ was thoroughly homogenized, the homogenate was serially diluted in sterile distilled water, and the dilutions were plated on blood agar (BBL Microbiology Systems, Cockeysville, Md.). The agar plates were incubated for 24 h at 37°C, and the colonies were enumerated. Results were expressed either as the mean ± the standard error of the mean (SEM) $\log_{10}$ CFU of *L. monocytogenes* per organ (four mice per treatment group) or as the $\log_{10}$ protection (reduction of CFU) of rIL-2-cultured LISC-treated versus control *L. monocytogenes*-infected mice. In experiments with an alternative infection challenge, clinical isolates of *Salmonella typhimurium*, *Streptococcus pyogenes*, and *Yersinia enterocolitica* (obtained from D. Stai and A. Sample, University of Wisconsin School of Veterinary Medicine, Madison) were processed in an identical manner, except that an appropriate challenge dose was used for each bacterial species.

Listeriocidal assay. The listeriocidal activity of rIL-2-cultured LISC was determined by use of a bactericidal assay that has been described in detail (13). In brief, 5×10^5 to 40×10^5 LISC were rotated (8 rpm) while being incubated for 2 h at 37°C with 10^5 *L. monocytogenes* in HBSS containing 10% pooled normal BDF_1 mouse serum. Afterwards, 0.1-ml samples were removed from each tube, serially diluted in sterile distilled water, and plated on blood agar. The plates were incubated at 37°C for 24 h, and the colonies were enumerated to determine the CFU of *L. monocytogenes*.

LISC cytotoxicity for macrophages. Thioglycolate-elicited peritoneal macrophages were chosen as a well-described macrophage population that lacks the capacity to kill ingested *L. monocytogenes* (2). Sterile thioglycolate medium was injected intraperitoneally (1.0 ml per mouse) 2 or 3 days before the mice were euthanized and their peritoneal exudate cells were harvested with 10 ml of Ca^{2+}- and Mg^{2+}-free HBSS with 0.01 M disodium EDTA. The macrophages were washed twice and resuspended in HBSS with 0.25% bovine serum albumin, and the suspension was added to individual wells (10^5 macrophages per well) of a nine-well tissue culture plate. The macrophages were allowed to adhere for 2 h at 37°C, and the nonadherent cells were removed by a warm PBS wash.

Both uninfected and *L. monocytogenes*-infected macrophages were used as target cells to assess the cytotoxicity of

rIL-2-cultured LISC. For infection of the macrophages, *L. monocytogenes* was opsonized with pooled normal mouse serum, added to the wells at a ratio of 20 listeriae per macrophage, and incubated at 37°C for 30 min. The monolayers were washed three times with warm RPMI 1640 tissue culture medium containing gentamicin (25 μg/ml). This procedure resulted in approximately 55% of the macrophages being infected, with an average of 4.4 listeriae per infected macrophage.

LISC were incubated with rIL-2 for 3 days, washed, and added to wells containing adherent peritoneal exudate macrophages at effector/target ratios of 0.1:1 to 5:1. The effector and target cells were coincubated for 18 h in RPMI 1640 with 10% fetal bovine serum and gentamicin (25 μg/ml) at 37°C in 5% CO_2. Macrophage lysis was quantitated by use of a previously described colorimetric assay (24). In brief, nonadherent cells were removed by washing of the monolayers three times, a 0.5% neutral red solution was added to the wells, and the monolayers were incubated for 1 h at 37°C. Then, the neutral red solution was removed, the cells were washed twice with warm PBS, and the intracellular neutral red was extracted with 0.05 M acetic acid in 0.5% sodium dodecyl sulfate. The optical density (OD) of the supernatants was quantified by use of a microELISA plate reader (Dynatech, Chantilly, Va.) at a wavelength of 550 nm. Controls included macrophages incubated in medium alone (spontaneous lysis) and macrophages lysed with distilled water (complete lysis). Results are expressed as percent specific lysis calculated as follows: [(OD of treatment wells − OD of spontaneous lysis control)/(OD of complete lysis control − OD of spontaneous lysis control)] × 100. Results are the means of three replicate wells for each treatment group.

Statistical analysis. Statistical analysis was performed by use of the two-tailed Student's *t* test for unpaired samples. Statistical significance was set at $P < 0.05$ for all comparisons.

RESULTS

Incubation of LISC with rIL-2 enhances the adoptive transfer of antilisteria resistance. We first examined the effect of 72 h of incubation with rIL-2 on the level of antilisteria resistance transferred by relatively small numbers (10^6) of LISC (Table 1). Although 10^6 untreated LISC transferred little resistance, 10^6 LISC that had been cultured for 72 h in the presence of 0.5 μg of rIL-2 per ml transferred a moderate level (0.75 $\log_{10}$) of antilisteria resistance. Incubation of LISC with rIL-2 for less than 72 h did not enhance the adoptive transfer of antilisteria resistance (data not shown). The protection was dependent on the LISC themselves rather than on a soluble mediator released in vitro, since i.v. injection of conditioned medium from an equivalent number of rIL-2-cultured LISC failed to increase antilisteria resistance in recipients (data not shown). Previous studies suggested that the in vivo administration of a low dose of rIL-2 enhanced the antitumor effect of tumor-infiltrating lymphocytes in human cancer patients (4) and prolonged the survival of antigen-specific T cells in mice (9). We similarly observed that the level of protection transferred by 10^6 LISC incubated for 72 h with rIL-2 was elevated by the i.v. administration of 0.6 μg of rIL-2 (Table 1), an rIL-2 dose 1/10 that shown previously to increase antilisteria resistance in vivo (18). In separate experiments, we showed that additional injections of rIL-2 did not significantly elevate antilisteria resistance (1.39 $\log_{10}$ protection with a single injection of 0.6 μg of rIL-2, compared with 1.66 $\log_{10}$ protection with two additional injections of 0.6 μg of rIL-2 at 1 and 2 days after *L. monocytogenes* challenge). A single in vivo administration of rIL-2, therefore, was used in all subsequent experiments.

TABLE 1. Enhancement by incubation with rIL-2 of the ability of LISC to adoptively transfer antilisteria resistance

LISC	rIL-2 in vitro[a]	rIL-2 in vivo[b]	$\log_{10}$ protection[c]
+	+	+	1.43 ± 0.20[d]
+	+	−	0.76 ± 0.15[d]
+	−	+	0.40 ± 0.14
+	−	−	0.00 ± 0.17
−	−	+	0.10 ± 0.18

[a] LISC (2×10^6 per ml) were cultured for 72 h in medium containing 0.5 μg of rIL-2 per ml per 10^6 LISC or in medium alone.

[b] At the end of the culture period, nonadherent cells were washed and then adoptively transferred into naive recipient mice that were challenged i.v. 2 h later with 5×10^4 *L. monocytogenes* and 0.6 μg of rIL-2.

[c] Mice were euthanized 3 days after challenge, and the $\log_{10}$ CFU of *L. monocytogenes* per spleen (four mice per group) were determined. Data are the mean ± SEM $\log_{10}$ reduction in CFU, compared with the CFU in untreated control *L. monocytogenes*-infected mice, in one representative experiment of three that were performed.

[d] $P < 0.05$, compared with control *L. monocytogenes*-infected mice and compared with each other.

Effects of time of incubation of LISC with rIL-2 on the transfer of antilisteria resistance. Maximal levels of protection were observed when LISC were incubated with rIL-2 for 3 to 10 days before adoptive transfer (Fig. 1); beyond 10 days, LISC rapidly lost their ability to transfer antilisteria resistance. In contrast, nonimmune spleen cells incubated with rIL-2 for 1 to 12 days could not transfer antilisteria resistance (Fig. 1). The capacity of rIL-2 to potentiate the transfer of resistance by LISC was abrogated when the rIL-2 was heat inactivated (100°C for 60 min) prior to addition to the LISC cultures (data not shown). This result indicates

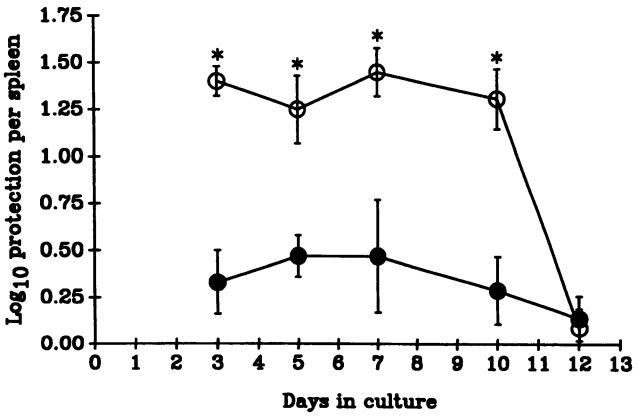

FIG. 1. Effect of time in culture with rIL-2 on the ability of LISC to transfer antilisteria resistance. Nonimmune spleen cells (●) or LISC (○) were cultured in medium containing rIL-2 (0.5 μg/ml). After the indicated number of days, cells were washed and adoptively transferred into naive recipient mice concomitantly with 5×10^4 *L. monocytogenes*. Mice were euthanized 3 days later, and the numbers of listeriae per spleen were determined. Results are expressed as the mean ± SEM $\log_{10}$ reduction in the numbers of viable *L. monocytogenes* per spleen (four mice per group) in four separate experiments. *, $P < 0.05$, compared with control *L. monocytogenes*-infected mice.

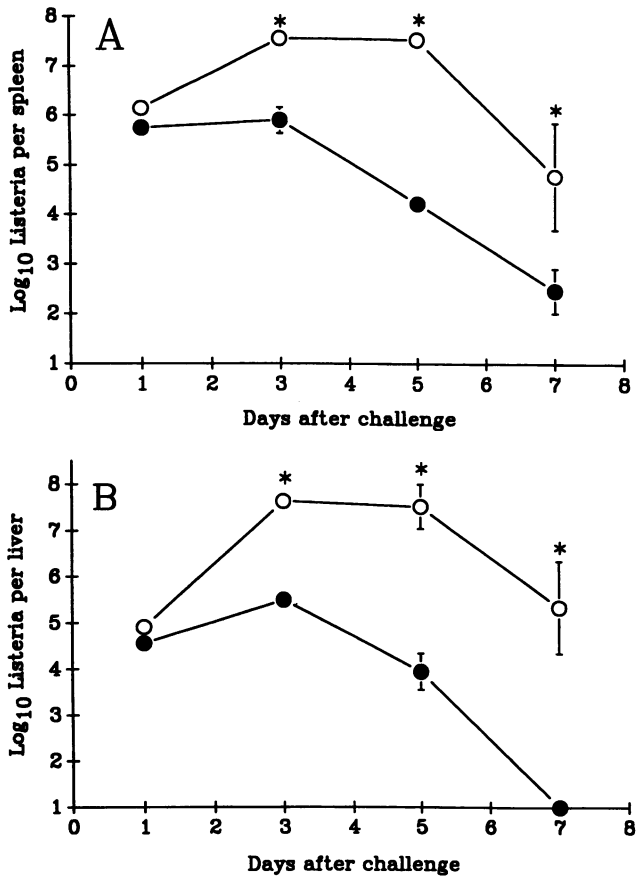

FIG. 2. Adoptive transfer of rIL-2-cultured LISC limits the severity of *L. monocytogenes* infection in mice. Results represent the mean ± SEM $\log_{10}$ CFU of *L. monocytogenes* recovered from the spleens (A) and livers (B) of infected mice (four mice per group) at the indicated times after challenge. Mice received either 10^6 rIL-2-incubated LISC (●) or vehicle (○). Control mice that died as a result of infection (two on day 5 and one on day 7) were assigned a value of 8.0 $\log_{10}$ CFU for the purpose of calculating the mean ± SEM $\log_{10}$ CFU of *L. monocytogenes* per spleen and liver on days 5 and 7. *, $P < 0.05$, compared with control *L. monocytogenes*-infected mice.

that it was the rIL-2 itself, rather than the minute amount of contaminating endotoxin present, that enhanced the transfer of resistance by LISC.

Transfer of rIL-2-cultured LISC reduces the numbers of *L. monocytogenes* without affecting their organ distribution in vivo. We next performed sequential examinations of *L. monocytogenes*-infected mice (Fig. 2). Mice that had received 10^6 LISC incubated for 72 h with rIL-2 at the time of bacterial challenge were compared with control *L. monocytogenes*-infected mice. By 3 days after challenge, substantial protection was detected in the spleens (Fig. 2A) and livers (Fig. 2B) of mice that received 10^6 rIL-2-cultured LISC. At later times, the bacterial burden was significantly reduced and the rate of bacterial clearance was accelerated in mice that had received 10^6 rIL-2-cultured LISC. In addition, none of the mice that had received LISC died, whereas some control mice died at later times (Fig. 2).

To exclude the possibility that the transfer of rIL-2-cultured LISC resulted in a redistribution of *L. monocytogenes* from the spleen and liver, we quantified the numbers of viable listeriae in various organs at 3 days after challenge,

TABLE 2. Effects of adoptive transfer of rIL-2-cultured LISC on the tissue distribution of *L. monocytogenes*

TIssue	$\log_{10}$ CFU of *L. monocytogenes* in[a]:	
	Mice receiving rIL-2-cultured LISC[b]	Control mice
Spleen	6.47 ± 0.22 (4)[c]	8.07 ± 0.11 (4)
Liver	5.92 ± 0.25 (4)[c]	7.77 ± 0.17 (4)
Kidney	2.36 ± 0.20 (2)[c]	4.13 ± 0.34 (4)
Lung	2.55 ± 0.54 (1)[c]	4.47 ± 0.37 (4)
Brain	None detected	2.68 ± 0.43 (2)
Blood	Bacteremic (1)	Bacteremic (4)

[a] Mice were challenged i.v. with 5×10^4 *L. monocytogenes* and euthanized 3 days later, and the mean ± SEM $\log_{10}$ CFU of *L. monocytogenes* per g of tissue (wet weight) were determined. The numbers of parentheses indicate the number of mice from which listeriae were recovered; a total of four mice were sampled. The means were calculated by assigning a value of 2.0 $\log_{10}$ CFU (limit of detection) to mice from which viable listeriae were not recovered. A 0.1-ml sample of serum was used for blood cultures.

[b] Mice received 10^6 rIL-2-cultured LISC plus 0.6 μg of rIL-2 i.v. 2 h before the *L. monocytogenes* challenge.

[c] $P < 0.05$, compared with control *L. monocytogenes*-infected mice.

the peak of the infection (Table 2). Adoptive transfer of rIL-2-cultured LISC provided a significant level of protection in all organs examined.

In vitro incubation with rIL-2 sustains the protection transferred by LISC. The substantial level of antilisteria immunity conveyed by the transfer of 5×10^7 freshly prepared LISC rapidly decays and is virtually absent by 2 days after the transfer (6). We observed that the maximal level of protection transferred by 10^6 rIL-2-cultured LISC plateaued for about 5 days and that protection then diminished to undetectable levels by 11 days after transfer (Fig. 3).

Transfer of rIL-2-cultured LISC does not increase resistance to other pathogenic bacteria. To determine whether the protection conferred by the transfer of 10^6 rIL-2-cultured LISC extended to other facultative intracellular pathogens, we performed experiments in which mice were challenged

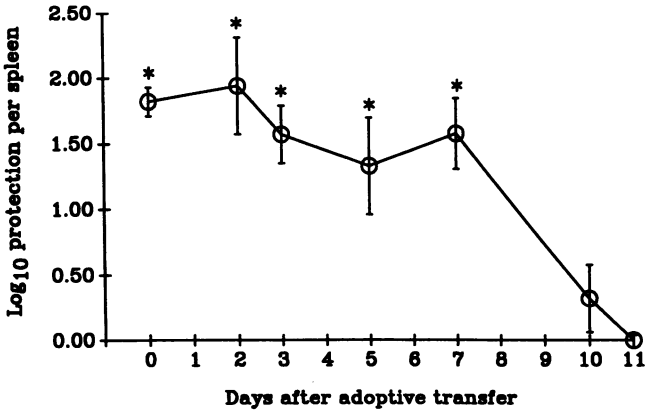

FIG. 3. Duration of antilisteria resistance transferred by rIL-2-incubated LISC. Mice were challenged with 5×10^4 *L. monocytogenes* at the indicated days after transfer of 10^6 rIL-2-cultured LISC. Mice were euthanized 3 days after bacterial challenge, and their spleens were removed to determine the $\log_{10}$ CFU of viable listeriae. Results are the mean ± SEM $\log_{10}$ reduction in CFU of viable listeriae, compared with the CFU in control *L. monocytogenes*-infected mice (four mice per group in each of three separate experiments). *, $P < 0.05$, compared with control *L. monocytogenes*-infected mice.

TABLE 3. Protection against *L. monocytogenes* but not *S. typhimurium* or *Y. enterocolitica* infection by transfer of rIL-2-cultured-LISC

Bacterial challenge[a]	LISC[b]	Log_{10} CFU of *L. monocytogenes* in[c]:	
		Spleen	Liver
L. monocytogenes	−	6.98 ± 0.16	7.31 ± 0.27
	+	5.59 ± 0.18[d]	5.13 ± 0.19[d]
S. typhimurium	−	4.88 ± 0.01	5.01 ± 0.05
	+	4.81 ± 0.02	5.10 ± 0.03
Y. enterocolitica	−	5.31 ± 0.27	5.13 ± 0.39
	+	5.06 ± 0.28	5.78 ± 0.42

[a] Mice were challenged i.v. with the indicated bacteria and euthanized 3 days later. Their spleens and livers were removed and processed to determine the CFU of viable bacteria as described in Materials and Methods. The challenge doses for *L. monocytogenes*, *S. typhimurium*, and *Y. enterocolitica* were 5×10^4, 3×10^4, and 2×10^4 viable bacilli, respectively.

[b] +, mice received 10^6 rIL-2-cultured LISC 2 h before bacterial challenge. In addition, 0.6 μg of rIL-2 was given i.v. concomitantly with the bacterial challenge.

[c] Results are the mean ± SEM log_{10} CFU (four mice per group) in one representative experiment of three that were performed.

[d] $P < 0.05$, compared with mice that did not receive rIL-2-cultured LISC.

with *S. typhimurium* and *Y. enterocolitica*. Mice that had received rIL-2-cultured LISC just prior to *L. monocytogenes* challenged displayed significant levels of protection, whereas mice challenged with either *S. typhimurium* or *Y. enterocolitica* were unprotected (Table 3). Separate experiments in which both higher and lower challenge doses of *S. typhimurium* and *Y. enterocolitica* were used confirmed that adoptive transfer of rIL-2-cultured LISC did not protect mice against challenge with these pathogens (data not shown). We also examined the effects of adoptive transfer of rIL-2-cultured LISC on the resistance of mice to *S. pyogenes*, a gram-positive bacterial pathogen that produces a hemolysin closely related to that of *L. monocytogenes* (17). Injection of a lethal challenge dose of *S. pyogenes* (5×10^8 CFU per mouse) resulted in the death of all rIL-2-cultured LISC-treated and control mice within 24 h.

The addition of $TGF\beta_1$ in vitro blunts the ability of rIL-2 to increase the protective activity of LISC. $TGF\beta_1$ is a known antagonist of IL-2-mediated cell activities (42). When 5.0 ng of $TGF\beta_1$ per ml was added within 48 h of the initiation of the LISC culture, it blocked the capacity of rIL-2 to enhance the antilisteria resistance transferred by LISC (Table 4, experiments 1 and 3 to 5). In separate experiments, $TGF\beta_1$ did not inhibit the level of antilisteria resistance transferred by 10^6 rIL-2-cultured LISC when it was added after 72 h of coincubation of LISC with rIL-2 (Table 4, experiment 2).

Characterization of rIL-2-cultured LISC cell populations. LISC incubated with rIL-2 for 3 days were assessed for their spontaneous proliferation in tissue culture medium to which no mitogen or antigen was added. LISC cultured for 60 h in medium alone demonstrated a significant uptake of [^{3}H]thymidine (11,081 cpm, compared with 478 cpm in nonimmune spleen cells), presumably because of their prior in vivo stimulation during *L. monocytogenes* infection. [^{3}H]thymidine uptake by LISC was elevated 11-fold (122,112 cpm) as a result of incubation with rIL-2 for 60 h in vitro; the addition of $TGF\beta_1$ (5 ng/ml) resulted in an intermediate level of [^{3}H]thymidine uptake (55,838 cpm). These results were reflected in flow cytometric analyses of forward and side-angle light scattering, which indicated that rIL-2-cultured LISC had many more large, apparently cycling cells than did LISC cultured in medium alone (data not shown). In keeping with the [^{3}H]thymidine data, the forward and side-angle light scattering profiles of LISC incubated with both rIL-2 and $TGF\beta_1$ were intermediate. As indicated in Table 5, the percentages of $CD4^+$ cells did not differ between LISC incubated with rIL-2 and LISC incubated with medium alone. However, the percentages of $CD8^+$ cells, γδ T cell receptor ($\gamma\delta$ TCR^+) cells, $B220^+$ cells and, to a lesser extent, NK-1.1^+ cells were increased as a result of incubation with rIL-2.

TABLE 4. Blocking by the addition of $TGF\beta_1$ during incubation with rIL-2 of the ability of LISC to transfer antilisteria resistance[a]

Expt	$TGF\beta_1$	Time added (h)	Log_{10} protection
1	+	0	0.46 ± 0.18[b]
	+	24	0.54 ± 0.20[b]
	+	48	0.83 ± 0.23[b]
	−		1.50 ± 0.15
2	+	0	0.17 ± 0.16[b]
	+	72	1.49 ± 0.15
	−		1.83 ± 0.16
3	+	0	0.61 ± 0.10[b]
	−		2.05 ± 0.26
4	+	0	0.49 ± 0.14[b]
	−		1.38 ± 0.23
5	+	0	0.90 ± 0.20[c]
	−		1.23 ± 0.04

[a] LISC were cultured with rIL-2 (0.5 μg/ml) for 3 days prior to adoptive transfer (4 days in experiment 2 only). $TGF\beta_1$ (5 ng/ml) was added to the cutlures at the indicated times after initiation of the cultures. The LISC were washed and injected i.v. into recipient mice, which were challenged i.v. 2 h later with 5×10^4 *L. monocytogenes* and 0.6 μg of rIL-2. Mice were euthanized 3 days later, and the log_{10} CFU of *L. monocytogenes* per spleen were determined as described in Materials and Methods. Results are the mean ± SEM log_{10} reduction in CFU (four mice per group), compared with the CFU in untreated control *L. monocytogenes*-infected mice.

[b] $P < 0.05$, compared with mice that had received rIL-2-cultured LISC not exposed to $TGF\beta_1$ in that experiment.

[c] $P = 0.16$, compared with mice that had received rIL-2-cultured LISC not exposed to $TGF\beta_1$ in that experiment.

TABLE 5. Flow cytometric analysis of rIL-2-cultured LISC

MAb	% Positively staining	
	LISC[a]	rIL-2-incubated LISC[b]
Anti-Thy-1.2	30 ± 9	62 ± 18
Anti-CD8	11 ± 8	34 ± 13
Anti-CD4	18 ± 4	20 ± 7
Anti-γδ T cell	11 ± 3	21 ± 10
Anti-NK-1.1	5 ± 3	9 ± 4
Anti-B220	23 ± 5	40 ± 3

[a] LISC were cultured in medium alone for 3 days. Results are the mean ± SEM in five separate experiments. Mice that had received 10^6 LISC and were then challenged 2 h later with 5×10^4 *L. monocytogenes* yielded 7.55 ± 0.11 log_{10} CFU of *L. monocytogenes* per spleen when euthanized 3 days later.

[b] LISC were cultured with rIL-2 (0.5 μg/ml) for 3 days. Results are the mean ± SEM in 20 separate experiments. Mice that had received 10^6 rIL-2-cultured LISC and were then challenged 2 h later with 5×10^4 *L. monocytogenes* yielded 5.90 ± 0.26 log_{10} CFU of *L. monocytogenes* per spleen when euthanized 3 days later.

TABLE 6. Effect of T cell subset depletion on the adoptive transfer of antilisteria resistance by rIL-2-cultured LISC[a]

MAb	Mean ± SEM $\log_{10}$ protection	
	Spleen	Liver
None	1.96 ± 0.21	2.30 ± 0.36
Anti-Ia^k	1.84 ± 0.17	1.92 ± 0.17
Anti-CD4	2.04 ± 0.08	1.93 ± 0.37
Anti-CD8	1.41 ± 0.25	1.61 ± 0.34
Anti-Thy-1.2 + anti-CD8 + anti-CD4[b]	1.09 ± 0.23[c]	1.53 ± 0.35[c]

[a] LISC were cultured with rIL-2 for 72 h. At the end of the culture period, T cell subpopulations were depleted with the indicated MAbs plus complement. The cells were washed and then adoptively transferred i.v. into recipient mice. These mice received 0.6 μg of rIL-2 concomitantly with 2×10^4 *L. monocytogenes* 1 h later. Mice were euthanized 3 days later, and the numbers of listeriae per spleen and liver were determined. Results are the mean ± SEM $\log_{10}$ reduction in CFU (four separate experiments), compared with the CFU in control *L. monocytogenes*-infected mice.

[b] Following this treatment there remained an average of 22% Thy-1.2^+ cells.

[c] $P < 0.05$, compared with mice that had received untreated rIL-2-cultured LISC.

Effects of T cell depletion on the adoptive transfer of antilisteria resistance by rIL-2-cultured LISC. To determine the role of $CD4^+$ and $CD8^+$ T cells in adoptive transfer by rIL-2-cultured LISC, we depleted these T cell populations by incubation with the appropriate MAbs and complement. Treatment of rIL-2-cultured LISC with an anti-Ia^k MAb (an irrelevant MAb) and complement or an anti-CD4 MAb and complement just prior to adoptive transfer did not reduce the level of protection transferred (Table 6). In contrast, rIL-2-cultured LISC treated with an anti-CD8 MAb and complement transferred significant protection, although it was diminished somewhat compared with that transferred by control rIL-2-cultured LISC. Treatment of rIL-2-cultured LISC with a combination of anti-Thy-1.2, anti-CD4, and anti-CD8 MAbs and complement significantly reduced but did not completely eliminate their ability to transfer antilisteria resistance. We can exclude the possibility that cytoplasmic constituents released by T cells during the depletion process conferred protection, because lysates of unseparated rIL-2-cultured LISC (disrupted by three freeze-thaw cycles) failed to transfer antilisteria resistance (data not shown).

Listeriocidal and cytotoxic activities of rIL-2-cultured LISC. To examine possible mechanisms by which rIL-2-cultured LISC transferred protection, we performed in vitro assays to assess the capacity of these cells to kill *L. monocytogenes* directly and to lyse *L. monocytogenes*-infected macrophages. In six separate experiments, rIL-2-cultured LISC, which were capable of conferring antilisteria resistance in vivo, failed to directly kill *L. monocytogenes* in vitro. *L. monocytogenes* incubated in medium alone increased by 1.12 ± 0.09 $\log_{10}$ CFU during a 2-h in vitro incubation, whereas *L. monocytogenes* coincubated with 120 LISC per listeria increased by 1.07 ± 0.11 $\log_{10}$ CFU during the same period. rIL-2-cultured LISC had high levels of cytotoxic activity against *L. monocytogenes*-infected macrophages (Fig. 4); however, uninfected macrophages were equally susceptible to cytolysis.

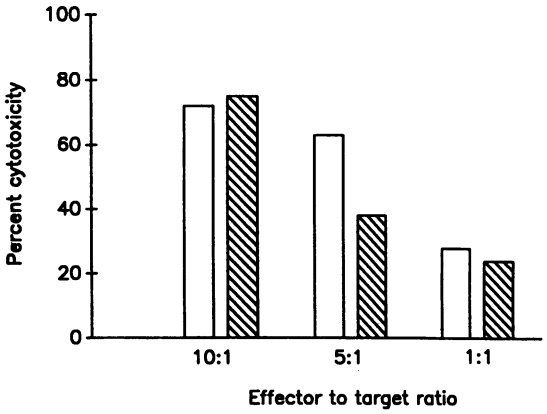

FIG. 4. Macrophage lysis by rIL-2-stimulated LISC in vitro. Nonadherent rIL-2-cultured LISC were added to adherent thioglycolate-elicited peritoneal exudate macrophages at the indicated effector/target ratios. Both *L. monocytogenes*-infected (□) and uninfected (▨) macrophages were used. LISC and macrophages were coincubated at 37°C for 18 h, and macrophage viability was quantified by colorimetric analysis of the uptake of neutral red as described in Materials and Methods. Data are from one representative experiment of six that were performed.

DISCUSSION

In a previous report we demonstrated that the exogenous administration of rIL-2 (6 μg per mouse) significantly enhanced antilisteria resistance in mice (18). Although we did not observe any obvious signs of toxicity in that study, rIL-2 therapy is associated with vascular leak syndrome in human cancer patients (1, 30, 48). In the present study, we examined the possibility that rIL-2 could be used to augment the protection mediated by the adoptive transfer of LISC. Our results indicated that 10^6 LISC, a number which is normally insufficient to protect mice against *L. monocytogenes* challenge, transfered significant protection when they were first incubated in vitro for at least 3 days with rIL-2 (Table 1). The addition of $TGF\beta_1$ during the first 72 h of culturing substantially reduced the effects of rIL-2. This result suggests that the continued presence of either rIL-2 itself or a $TGF\beta_1$-sensitive cascade of rIL-2-induced cytokines and cellular events is necessary for the maximal development of the antilisteria capacity of LISC in vitro. Mice that received rIL-2-incubated LISC experienced a decreased bacterial burden and an accelerated rate of bacterial clearance from their spleens and livers. Significant levels of antilisteria resistance were maintained for about 1 week after the adoptive transfer of 10^6 rIL-2-cultured LISC. In contrast, antilisteria resistance transferred by 5×10^7 freshly isolated LISC typically lasts less than 24 h (3). Bishop and Hinrichs (6) reported that the in vitro incubation of LISC with concanavalin A before adoptive transfer resulted in antilisteria resistance being sustained for up to 8 weeks. Others, however, observed little protection when LISC were cultured with concanavalin A before adoptive transfer (43). These studies suggest that ex vivo culturing may, under certain conditions, extend the duration and increase the magnitude of protection transferred by LISC.

The adoptive transfer of 10^6 rIL-2-cultured LISC protected mice against challenge with *L. monocytogenes* but not against challenge with *S. typhimurium* and *Y. enterocolitica* or against a lethal challenge with *S. pyogenes*, which produces a hemolysin that shares considerable homology with the hemolysin of *L. monocytogenes* (17). The lack of activity against other intracellular pathogens suggested that the protective cells within the rIL-2-cultured LISC population were not nonspecific effector cells, such as NK or lympho-

kine-activated killer (LAK) cells. Several lines of unpublished evidence indicate that antigen-specific $CD4^+$ (24, 33) and $CD8^+$ (8, 14, 22, 31) T cells can both contribute to antilisteria resistance. In the present study, the depletion of $CD4^+$ cells from the rIL-2-cultured LISC did not alter their capacity to transfer antilisteria resistance, whereas the depletion of $CD8^+$ T cells resulted in a modest reduction (approximately 0.55 to 0.69 $\log_{10}$ CFU) in the level of protection transferred. The combined depletion of $CD4^+$, $CD8^+$, and Thy-1.2^+ cells significantly decreased the ability of rIL-2-cultured LISC to transfer antilisteria resistance, although a substantial level of protection remained. These data suggest that $CD8^+$ T cells contribute in part to the antilisteria resistance transferred by rIL-2-cultured LISC, although the majority of the resistance appears to be dependent on some other cell type.

Incubation of mononuclear cells with IL-2 results in the generation of LAK cells and NK cells (34) which secrete high levels of tumor necrosis factor alpha, interleukin-1α, and gamma interferon (30). Each of these cytokines has been reported to augment antilisteria resistance (7, 12, 15, 19, 44). In addition, LAK and NK cells are cytotoxic for mononuclear phagocytes, particularly those infected with various intracellular pathogens (41). We considered the possibility that LAK cells or NK cells were the cell types responsible for the transfer of antilisteria resistance. We believe that this is unlikely, however, because of the low numbers of rIL-2-incubated LISC transferred (10^6 per mouse) and because incubation of nonimmune spleen cells with rIL-2 for up to 12 days in vitro resulted in many cells that morphologically resembled LAK cells but lacked the ability to transfer antilisteria resistance. Although NK cell activity and nonspecific immunity are increased following infection with *L. monocytogenes* (20, 26) or injection of *L. monocytogenes* cell walls (39), the relative importance of NK cells in antilisteria resistance is controversial. It has been reported that the depletion of NK cell activity does not impair the adoptive transfer of antilisteria resistance by LISC (31). NK cells produce substantial amounts of gamma interferon during the early stage of *L. monocytogenes* infection (16, 37); however, the importance of this response in antilisteria resistance is not clear. In one report, the depletion of NK cells with anti-asialo GM_1 antibodies had no effect (37) on resistance to i.v. inoculation of *L. monocytogenes*, whereas other investigators reported that the splenic clearance of *L. monocytogenes* was enhanced after anti-asialo GM_1 antibody treatment (46). One recent report indicated a critical role for NK cells during the early response to *L. monocytogenes* infection; however, that study used a subcutaneous route of inoculation (16). We found that the administration of an anti–NK-1.1 MAb to mice impaired the ability of their spleen cells to lyse YAC-1 cells in vitro (NK activity) without altering antilisteria resistance in vivo (unpublished observations).

Antigen-specific $\gamma\delta$ TCR^+ cells have been shown to accumulate in granulomatous lesions caused by various infectious agents (22, 36). One report demonstrated that $\gamma\delta$ TCR^+ cells emigrate into the peritoneal cavities of mice soon after intraperitoneal injection with *L. monocytogenes*, suggesting that they may be involved in host defense against *L. monocytogenes* (38). In addition, many $\gamma\delta$ TCR^+ cell lines produce cytokines, such as interleukin-2 and gamma interferon, that are known to contribute to antilisteria resistance (11, 40). Table 5 demonstrates that the in vitro incubation of LISC with rIL-2 increased the percentage of $\gamma\delta$ TCR^+ cells. Because the available anti-$\gamma\delta$ T cell MAb is ineffective at depleting rIL-2-cultured LISC of $\gamma\delta$ TCR^+ cells; however, we could not directly assess their contributions to the transfer of antilisteria resistance.

Two possible effective mechanisms for the protective activity of rIL-2-cultured LISC, direct killing of *L. monocytogenes* (27) and lysis of *L. monocytogenes*-infected macrophages (14), were examined in vitro. The data obtained allow us to exclude the first possibility, because rIL-2-cultured LISC had no detectable listeriocidal activity at effector/target ratios of up to 240 LISC per bacterium. Although rIL-2-cultured LISC demonstrated high levels of cytolytic activity, they lysed uninfected and *L. monocytogenes*-infected macrophages equally well. In addition, significant protection after the transfer of rIL-2-cultured LISC was not detected until 3 days after *L. monocytogenes* challenge (Fig. 2). These findings, together with the relatively low numbers of cells transferred (10^6), led us to conclude that the direct lysis of *L. monocytogenes*-infected macrophages was not a major effector mechanism. Instead, it seems more likely that the increased antilisteria resistance resulted from the ability of rIL-2-cultured LISC to enhance the immune response of the recipient rather than from direct listeriocidal activity or lysis of infected macrophages.

In summary, this study indicates that rIL-2 can be used to augment adoptive immunotherapy of bacterial infection. Although the principal cell type responsible for protection was not definitively identified, protection appeared to be largely $CD4^+$ cell independent and only partly dependent on $CD8^+$ cells. Further analysis will be required to define the contribution of $\gamma\delta$ TCR^+ cells and perhaps other cell types to the adoptive transfer of antilisteria resistance by rIL-2-cultured LISC.

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