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Enhanced Adoptive Transfer of Immunity to *Listeria monocytogenes* After In Vitro Culture of Murine Spleen Cells with Concanavalin A

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In vitro incubation of spleen cells with T-cell mitogens has been shown to augment cytotoxic and cytolytic T-cell function. The plant lectins Concanavalin A and phytohemagglutinin were employed in a similar fashion to investigate their abilities to enhance cell-mediated immunity to the microbial pathogen *Listeria monocytogenes*. Spleen cells from immune mice were incubated in vitro with Concanavalin A or phytohemagglutinin before passive transfer into normal recipients. Results indicated a 10- to 100-fold enhancement in the ability of these cells stimulated in vitro to transfer antilisterial resistance, as assayed by changes in 50% lethal dose values and enumeration of splenic bacterial proliferation.

Listeria monocytogenes is a facultative intracellular bacterial parasite which replicates within the mononuclear phagocytic cells of an infected host (10). Recovery from experimentally induced infections with *L. monocytogenes* results in high levels of immunity to this pathogen. Various preparations of nonviable *L. monocytogenes*, utilized as vaccines, induce serum-contained antibody but do not induce acquired resistance to subsequent challenge with viable *L. monocytogenes* (10, 11). The immunity that develops to *L. monocytogenes* depends on the bactericidal activity of activated macrophages (10, 11, 13). These macrophages are activated by lymphocyte mediators after exposure of antigen-specific lymphocytes to *L. monocytogenes* antigens (12, 16). Passive transfer experiments have identified these antigen-specific lymphocytes as T cells (2, 9, 14). The generation of these specific antilisterial immune lymphocytes apparently requires an in vivo response to viable organisms.

In certain systems of cell-mediated immunity, effector T-cell activities can be developed or enhanced by in vitro exposure to selected T-cell mitogens. The polyclonal T-cell mitogenic activity of the plant lectin Concanavalin A (ConA) has been previously established (1, 5, 22). In functional studies, it has been found that in vitro culture of normal murine spleen cells with ConA enhances the primary cytotoxic response to allogenic target cells (6, 23). Addition of ConA or phytohemagglutinin (PHA), another plant lectin mitogenic for T cells, to long-term primary mixed lymphocyte cultures stimulates the specific development of cytolytic activity (23). Similarly, in vitro incubation of alloantigen-primed murine spleen cells with ConA activates specific secondary cytotoxic lymphocyte responses (3, 6, 20). In a tumor rejection model, spleen cells from mice primed with syngeneic tumor cells are specifically activated into secondary cytotoxic effector cells when incubated in vitro with ConA or PHA (4). Effector cell function is also developed in an autoimmune disease model, in which it has been observed that in vitro ConA stimulation of spleen cells obtained from rats exhibiting clinical experimental allergic encephalomyelitis appears to be a necessary in vitro step for the successful passive transfer of clinical disease to syngeneic recipients (7, 17, 18).

These studies of lectin-enhanced T-cell function have evaluated cytolytic or cytoxic activity. Although direct T-cell antilisterial toxicity has not been detected in this antimicrobial model of cell-mediated immunity, the enhancement of other functional T-cell activities after in vitro lectin stimulation prompted us to evaluate the role of T-cell mitogens in antilisterial immunity. It is the purpose of this communication to report the conditions required to enhance the passive transfer of specific resistance to *L. monocytogenes*. We have found that, depending on the level of donor immunity, in vitro culture of spleen cells with the plant lectins ConA or PHA dramatically enhanced the adoptive transfer of cell-mediated immunity to this microbial pathogen.

MATERIALS AND METHODS

Mice. Adult female BALB/c mice, 8 to 12 weeks old, were obtained from a colony maintained by the Department of Bacteriology and Public Health, Washing-

ton State University. Mice were housed under standard conditions and offered feed and water ad libitum.

Bacteria. *L. monocytogenes* strain 10403 serotype 1 (obtained from M. L. Gray, Montana State University) has been maintained in a virulent state by repeated passage in mice and has a 50% lethal dose (LD_{50}) of approximately 4.5×10^3 bacteria for BALB/c mice.

Reagents. Brain heart infusion (BHI) broth, BHI agar, and Proteose Peptone were obtained from Difco Laboratories. *Escherichia coli* (0111:B4) lipopolysaccharide (LPS; Difco) was rehydrated at 10 mg/ml in sterile phosphate-buffered saline and stored frozen. ConA (Sigma Chemical Co.) and PHA (Burroughs Wellcome Co.) were stored frozen at 1 mg/ml and 100 μg/ml, respectively, in RPMI 1640 medium (GIBCO Laboratories). Monoclonal anti-Thy 1.2 antibody was kindly provided by W. C. Davis, College of Veterinary Medicine, Washington State University. Low-toxicity-M rabbit complement was obtained from Cedar Lane Laboratories Ltd.

Infection of mice. Bacterial suspensions for immunization or challenge were prepared by inoculating 5 ml of BHI broth with *L. monocytogenes*. After being incubated for 18 h at 37°C, this culture broth was added to 50 ml of BHI broth and incubated another 4 h in a 37°C rotary shaker water bath. Tenfold dilutions of this log-phase culture were prepared in 1% peptone for subsequent intravenous (i.v.) injection of mice. Approximately 0.1 to 0.5 LD_{50} and 5.0 LD_{50} were utilized for primary and secondary immunizations, respectively.

Passive transfer of cells. Spleen cell suspensions in RPMI 1640 medium were prepared from normal or immune syngeneic donors. Immune donors were sacrificed 14 days after their last immunization. Recipient mice received 10^7 viable cells i.v. either before or after in vitro culture. Numbers of viable cells were determined by nigrosin dye exclusion. Four days after passive transfer of cells, mice were challenged with *L. monocytogenes*.

In vitro cultures. Spleen cells (2×10^6 viable cells per ml) were cultured in 50-ml volumes in 75-cm^2 tissue culture flasks for 3 days at 37°C in a humidified 5% CO_2–95% air incubator. Culture media consisted of RPMI 1640 medium supplemented with 5% fetal calf serum (Flow Laboratories), 2×10^{-5} M 2-mercaptoethanol (Sigma), 100 U of penicillin per ml, and 100 μg of streptomycin per ml. Cultures also included the presence or absence of the mitogens ConA, PHA, or LPS. After 3 days, cells cultured in vitro were collected, washed three times by centrifugation, and injected into normal recipient mice.

LD_{50} determinations. Groups of five mice each were injected i.v. with fivefold dilutions of log-phase *L. monocytogenes*. Mice were observed for 10 days, after which time no more deaths occurred. The LD_{50} was calculated by the method of Reed and Muench (19).

Enumeration of bacteria in spleens. At specified times after i.v. challenge with *L. monocytogenes*, mice were sacrificed and their spleens were aseptically extracted. The spleens were homogenized (Ultra-Turrax, Tekmar Co.), the homogenates were diluted tenfold in 1% peptone, and the dilutions were plated on BHI agar. The numbers of colony-forming units (CFU) per milliliter of spleen homogenate were determined after incubation of the plates for 48 h in a 37°C air incubator.

RESULTS

ConA-enhanced transfer of antilisterial resistance. The effect of an in vitro culture period on the subsequent ability of spleen cells to transfer resistance to a viable *L. monocytogenes* challenge is shown in Table 1. Spleen cells obtained from either normal or immune mice were cultured in vitro with 1.0 μg of ConA per ml before passive transfer. LD_{50} values were determined for groups of mice receiving normal or immune spleen cells without in vitro culture or cultured in vitro with or without ConA. Results of these experiments indicate that exposure to ConA in vitro enhanced the ability of spleen cells from immunized mice to transfer antilisterial resistance to normal mice. Spleen cells from normal mice cultured with ConA did not transfer resistance. An additional study demonstrated that treatment of ConA stimulated immune spleen cells with α-methyl mannoside before passive transfer did not inhibit the transfer of this resistance (data not shown).

Effect of donor immunization on adoptive transfer of antilisterial resistance. To establish the role of the donor in ConA-enhanced transfer, we made LD_{50} determinations for mice which had received cells from donors subjected to primary or primary and secondary immunizations with viable *L monocytogenes*. Several experiments were performed in which these cell populations remained untreated before passive transfer or were cultured in vitro with or without ConA.

The summarized results (Table 2) show that spleen cells from donor mice receiving two immunizations, rather than one, were more effective in adoptive transfer of resistance. Spleen cells from primarily immunized donors, when cultured in vitro with ConA, were as effective in transferring resistance as noncultured spleen cells from secondarily immunized

TABLE 1. Adoptive transfer of immunity to *L. monocytogenes* after in vitro culture of spleen cells

Source of transferred cells	In vitro culture	Mitogen (μg/ml)	LD_{50} protection[a]
Normal mice	−		1.0
	+	None	1.0
	+	ConA (1.0)	2.1
Immune mice[b]	−		3.7
	+	None	7.5
	+	ConA (1.0)	24.9

[a] All recipient mice received 10^7 viable spleen cells; the LD_{50} protection was determined by dividing the LD_{50} value for recipient mice by the LD_{50} value of normal mice (4.7×10^3 CFU).

[b] Mice received both primary and secondary immunizations.

TABLE 2. Influence of donor immunization on adoptive transfer of antilisterial resistance

Donor immunization	In vitro culture[a]	Range of LD_{50} protection[b]
1°	–	<5
1° + 2°	–	3–17
1°	+	2–10
1° + 2°	+	20–240

[a] 1.0 μg of ConA per ml.
[b] Determined as specified in Table 1; ranges were determined from results of four or more experiments.

donors. The most effective transfer of resistance occurred with ConA-conditioned spleen cells obtained from secondarily immunized donors.

Bacterial proliferation in recipient mice. Bacterial proliferation assays, in which spleens were used as indicator organs, have been previously utilized to assess passive transfer of antilisterial resistance (9, 12, 13). We utilized this assay to determine if splenic bacterial proliferation in mice receiving cell populations cultured in vitro were similar to those in mice receiving nonconcultured cell populations. The number of *L. monocytogenes* found in the spleens of these recipient mice, as well as in the spleens of normal and immune mice, were determined daily over a 3-day period. Results of this assay (Fig. 1) indicated that the largest difference among experimental groups existed at 48 h. This time point was therefore utilized in all further assays of splenic bacterial proliferation.

Comparison of LD_{50} determinations with splenic bacterial proliferation assay in terms of assessment of adoptive transfer of resistance. The results of LD_{50} determinations are assumed to be comparable to those of bacterial clearance assays in studies of passive transfer of antilisterial immunity. To verify this assumption for cells conditioned in vitro, we directly compared these two assays to determine if they would provide the same relative information on the ability of spleen cell populations to transfer protection against a viable *L. monocytogenes* challenge. Results of this comparision (Table 3) show that a decrease in splenic bacterial proliferation was directly related to an increase in the LD_{50} value in groups of mice exhibiting increased active or passive resistance to *L. monocytogenes*. The splenic bacterial proliferation assay was selected for further passive transfer studies because it provides the same information yet requires smaller numbers of donor and recipient mice per experiment.

Effect of LPS and PHA stimulation of primed spleen cells on adoptive transfer of antilisterial resistance. The enhancement of passive transfer activity that is seen after culture of immune spleen cells with ConA prompted us to evaluate two additional mitogens for similar activity. The bacterial-derived mitogen LPS and the plant lectin PHA were compared with ConA in terms of their ability to enhance transfer of resistance to *L. monocytogenes* after similar in vitro culture conditions. Spleen cells from immune donors were cultured in vitro without mitogen or

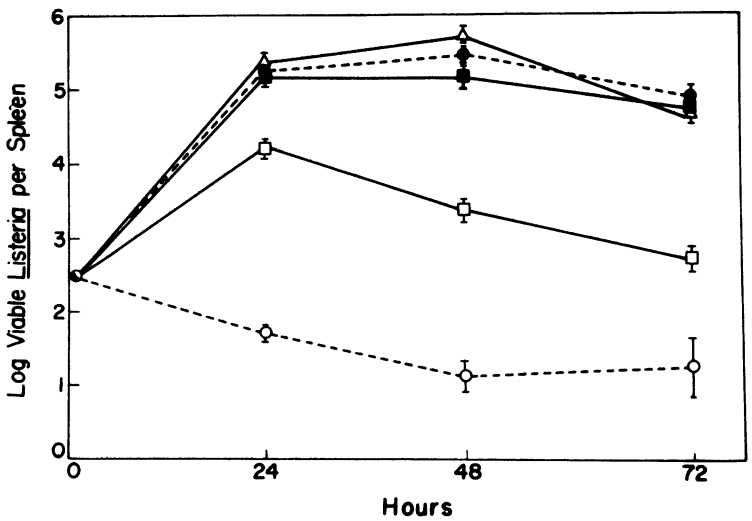

FIG. 1. Log_{10} viable bacteria per spleen in normal (●) or immune (○) mice, in mice receiving 10^7 viable spleen cells from immune donors (△), and in mice receiving 10^7 viable spleen cells from normal (■) or immune (□) mice after in vitro culture of transferred cells in 1.0 μg of ConA per ml. All mice were challenged i.v. with 3.0×10^3 *L. monocytogenes*. Each point represents the mean (± standard error of the mean) of five mice.

TABLE 3. Correlation of LD_{50} protection and splenic bacterial counts in adoptive transfer recipients after *L. monocytogenes* challenge

Group	In vitro[a] culture	LD_{50} protection[b]	Log_{10} CFU/spleen at 48 h[c]
Normal mice		1.0	8.71 (±0.15)
Immune mice		103.3	3.67 (±0.33)
Adoptive transfer recipients[d]			
Immune cells	−	2.2	8.13 (±0.10)
Immune cells	+	79.0	5.19 (±0.22)
Normal cells	+	1.5	8.71 (±0.18)

[a] 1.0 μg of ConA per ml.
[b] Determined as specified in Table 1.
[c] Means (±SEM) of five mice per group; all mice received an i.v. challenge of 1.7×10^5 CFU.
[d] All mice received 10^7 viable cells before challenge.

with LPS, PHA, or ConA before passive transfer into normal recipient mice. Results of this experiment (Table 4) show that cells cultured with PHA but not LPS exhibited an exhanced passive transfer activity similar to that found for cells cultured with ConA.

Titration of optimal concentrations of ConA and PHA which provide enhanced adoptive transfer of antilisterial immunity. Spleen cells obtained from immune donors were cultured with various concentrations of ConA or PHA before passive transfer to normal mice. Splenic bacterial proliferation was determined for each recipient group 48 h after *L. monocytogenes* challenge (Table 5). In vitro ConA and PHA concentrations that caused equivalent transfer after culture were determined to be 0.5 to 2.5 and 2.5 to 5.0 μg/ml, respectively.

T cells are required for enhanced transfer of antilisterial immunity after in vitro culture. The cells responsible for the adoptive transfer of antilisterial immunity have been previously classified as T cells (2, 9, 14). To establish if the transfer of immunity remains dependent on T-cell activity, we treated spleen cells conditioned in vitro from immune donors with monoclonal anti-Thy 1.2 antibody, complement, or both before passive transfer to normal recipients.

TABLE 4. Adoptive transfer of immunity to *L. monocytogenes* after in vitro culture of immune donor spleen cells with or without mitogens

In vitro culture	Mitogen (μg/ml)	Log_{10} CFU/spleen at 48 h[a]
−		5.77 (±0.14)
+	None	5.23 (±0.10)
+	LPS (10.0)	5.82 (±0.16)
+	PHA (2.5)	3.48 (±0.14)
+	ConA (1.0)	3.39 (±0.17)

[a] Means (±SEM) of five mice per group; all mice received i.v. challenge of 4.1×10^4 CFU.

Results of this experiment (Table 6) show that enhanced transfer activity was abrogated after treatment with anti-Thy 1.2 antibody and complement. This indicates that T cells were responsible for transfer activity after in vitro culture with ConA.

DISCUSSION

We have shown in this study that in vitro culture of primed spleen cells in the presence of ConA or PHA resulted in an enhancement of the antilisterial activity of these cells, as measured by adoptive transfer experiments. This enhancement varied depending on the immunization history of the spleen cell donor animals; it ranged from 10- to 100-fold (Table 2). In some experiments, antilisterial resistance in adoptively immunized animals receiving as low as 10^7 spleen cells stimulated in vitro was comparable to actively immunized animals (Table 3). The thymus dependent (T-cell) classification of these mitogen-stimulated spleen cells responsible for adoptive transfer of this resistance was verified, since treatment of these cells after in vitro culture with monoclonal anti-Thy 1.2 antibody and complement abrogated the transfer of this resistance (Table 6). These results are in general agreement with the observations of mitogen-enhanced functional T-cell activities in cytolytic and cytotoxic cell-mediated immune systems (3, 4, 20).

The nature of the in vitro events that lead to enhancement of adoptive transfer of immunity after culture with ConA or PHA can not be readily determined from this study. The fact that the effective dose range of ConA- or PHA-mediated transfer occurs at peak mitogenic concentration, as assayed by lymphocyte transformation (data not shown), implies that cell division or differentiation is required for acquisition of enhanced transfer capability. This would indicate that the *Listeria*-immune T cell is a

member of the ConA- and PHA-responsive T-cell population or that it is dependent on such a responsive population for its enhanced activity. The T-cell role in antilisterial immunity has previously been established (2, 9, 14), and our demonstration that enhanced activity after culture is a property of or dependent on cells bearing the Thy 1.2 antigen is consistent with these previously reported observations.

One explanation of the mitogen-induced enhancement seen in these studies is that ConA stimulation in vitro simply enlarged the population of *Listeria*-immune T cells. This direct stimulation and implied numerical increase is difficult to explain unless one assumes that *Listeria*-immune cells are more responsive to ConA stimulation or that these *Listeria*-immune cells exist as a major population of ConA-responsive cells in the spleens of the donor animals. If one-to-one comparisons for cell transfer are to be made, then this argument would have to explain the 100-fold increase in transfer activity seen after in vitro culture of immune spleen cells. Polyclonal stimulation by ConA is therefore difficult to reconcile with the dramatic enhancement of transfer activity observed after culture. However, the fact that spleen cells from secondarily immunized donors provide greater transfer activity after culture (Table 2) may support a direct-stimulation hypothesis.

It is possible that the effectiveness of the in vitro culture period is related to the regulatory events that are indicated in immunity to *L. monocytogenes*. The acquired resistance that develops to *L. monocytogenes* is apparently of short duration. Immunity following active immunization is evident for 2 to 3 months (8, 10). Passively acquired resistance is even more transient, persisting but a few days (16). Previous investigations have determined that the splenic T cells responsible for passive transfer of antilisterial resistance provide optimal protection when collected immediately after the clearance and destruction of viable organisms by the phagocytic organs of sublethally challenged donors (9, 12). Thereafter, the capability to adoptively immunize normal recipients rapidly declines until at about 3 weeks after immunization, when one organ equivalent of primed spleen cells no longer protects recipient mice against a lethal challenge (15). These observations suggest that the effector T cells responsible for adoptive immunization are short lived or highly regulated by suppressive mechanisms or both.

If suppressor mechanisms prove to be involved in the regulation of immunity to *L. monocytogenes*, it is possible that the lectins ConA and PHA could function to override any potential suppressive regulation of these effector cells or their precursors, resulting in cell differentiation, increased effector cell numbers, or both. It has been proposed that the in vitro culture step serves to allow differentiation to occur (4, 7, 20), promoting development of effector cells to a stage such that direct suppressor regulation is no longer effective (7). Therefore in this *Listeria* study, cells conditioned in vitro may lose complementary regulatory circuits or may be driven to a point of differentiation at which regulation is less effective. Investigations are continuing in our laboratory to differentiate between the various mechanisms that regulate the expression of immunity in this antimicrobial system. We hope

TABLE 5. Concentrations of ConA and PHA for in vitro culture which provide optimal enhancement of adoptive transfer of immunity to *L. monocytogenes*

Expt	Concn of mitogen (μg/ml)	Log_{10} CFU/spleen at 48 h[a]
1[b]	0.01	7.36 (±0.14)
	0.05	7.30 (±0.11)
	0.10	7.15 (±0.07)
	0.50	3.97 (±0.37)
	1.00	3.95 (±0.41)
	2.50	3.79 (±0.37)
	5.00	6.85 (±0.23)
2[c]	0.01	5.46 (±0.08)
	0.05	4.84 (±0.09)
	0.10	5.30 (±0.13)
	1.00	4.54 (±0.06)
	2.50	3.94 (±0.11)
	5.00	4.11 (±0.13)
	ConA (1.00)	3.88 (±0.07)

[a] Means (±SEM) of five mice per group.
[b] Mice received i.v. challenge of 3.6×10^4 CFU; log_{10} counts at 48 h for normal and immune mice were 7.55 (±0.09) and 1.71 (±0.25), respectively. The mitogen used was ConA.
[c] Mice received i.v. challenge of 2.0×10^3 CFU; log_{10} counts at 48 h for normal and immune mice were 5.19 (±0.09) and 1.74 (±0.08), respectively. The mitogen used was PHA, except where noted otherwise.

TABLE 6. Treatment of ConA-stimulated immune spleen cells which enhance adoptive transfer of immunity to *L. monocytogenes* with anti-Thy 1.2 antibody

Treatment after in vitro culture[a]	Log_{10} CFU/spleen at 48 h[b]
None	4.98 (±0.11)
Complement	5.09 (±0.12)
Anti-Thy 1.2	5.39 (±0.11)
Complement + anti-Thy 1.2	6.94 (±0.16)

[a] Each group of ConA-stimulated spleen cells was incubated for 30 min at 37°C.
[b] Means (±SEM) of five mice per group; all mice received i.v. challenge of 2.5×10^4 CFU; log_{10} bacterial counts at 48 h for normal and immune mice were 6.87 (±0.18) and 1.89 (±0.19), respectively.

the plant lectins ConA and PHA will be useful in the study of the onset and regulation of cell-mediated immunity to *L. monocytogenes* and other intracellular microbial pathogens.

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