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Cloning and Characterization of T-Cell-Reactive Protein Antigens from *Listeria monocytogenes*

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To explore the molecular basis of the T-cell-mediated immune response to *Listeria monocytogenes*, we cloned and expressed listerial antigens in *Escherichia coli* using the lambda-ZAP bacteriophage and Bluescript plasmid vectors. A two-stage screening strategy was implemented to identify T-cell-reactive antigens; the first stage involved antibodies or oligonucleotide probes and the second stage was based on assays for T-cell activation. A library of genomic DNA from *L. monocytogenes* was generated in lambda-ZAP, and then antigens were detected in infected cells with a polyclonal rabbit anti-*L. monocytogenes* antiserum and an *L. monocytogenes*-specific monoclonal antibody. Also, synthetic oligonucleotide probes corresponding to the structural gene for listeriolysin O (LLO) were used to screen the recombinant DNA library. In each case, positive isolates were evaluated for T-cell antigenicity by measuring antigen-induced interleukin-2 production by polyclonal T cells taken from *L. monocytogenes*-immune mice. Phage clones were subcloned and expressed in the Bluescript plasmid and tested further for antigenic activity and LLO expression. Using this screening strategy, we successfully identified bacterial clones producing recombinant listerial antigens which activate *L. monocytogenes*-immune T cells in vitro. Antigens operative in the T-cell response during infection with *L. monocytogenes* include LLO, 62- and 39-kilodalton proteins, and other poorly defined bacterial surface components. We also found that high concentrations of recombinant LLO inhibited macrophage-mediated antigen presentation. These results are discussed in terms of the multiple functions of LLO as a virulence factor, inhibitor of antigen presentation, and potent antigen in the T-cell response to *L. monocytogenes*. These studies represent the first step toward a genetic definition of the antigens recognized in immune defense to *L. monocytogenes*.

Listeria monocytogenes is a gram-positive, facultative, intracellular pathogen which is capable of survival and replication in macrophages. Its transmission in humans is frequently linked to contaminated foods (3, 11, 32). Listeriosis can be fatal in elderly and immunocompromised individuals (3, 29). In pregnant females, the bacteria can replicate uncontrollably at the fetoplacental interface, an area of putative local immunosuppression (31).

Murine listeriosis is a well-studied model of cell-mediated immunity. While a strong antibody response is made to a spectrum of cell surface proteins in addition to the secreted listeriolysin O (LLO), a major virulence factor, these antibodies are not sufficient to confer protection against challenge with *L. monocytogenes*. Instead, a protective immune response is mediated by T cells (1, 17, 22, 29). The involvement of both L3T4+ and Lyt2+ T cells in immunity to *L. monocytogenes* was first shown by Kaufmann et al. (16, 17). Similar observations were made by others (6) using in vitro-stimulated T-cell subsets.

Production of LLO is thought to be necessary for intracellular growth of this pathogen and the generation of protective immunity (4, 5, 12, 30). All strains isolated from clinical infections are beta-hemolytic, and transposon mutagenesis of the structural gene of LLO by three independent groups has resulted in an avirulent phenotype (12, 15, 30). Production of LLO has been shown to be associated with impairment of the antigen presentation function of macrophages, and this effect may contribute to virulence (8). The gene encoding LLO, *hlyA*, has been cloned and sequenced (25).

Although the mechanisms of virulence and cell-mediated immunity to *L. monocytogenes* have been well studied, very little is known about the molecular nature of the antigens to which this immunity is directed. The definition of such antigens is crucial to understanding the mechanism by which protective immunity to *L. monocytogenes* is generated. In this communication, we report the cloning of antigens from *L. monocytogenes* in *Escherichia coli* and the isolation of recombinant clones which stimulate T cells from *L. monocytogenes*-immune mice. The multiple functions of recombinant LLO as a membrane-active cytolysin, T-cell-reactive antigen, and inhibitor of antigen presentation are discussed.

MATERIALS AND METHODS

Bacterial strains. *L. monocytogenes* serotype 1/2a (ATCC 43251) was grown in brain heart infusion broth at 37°C with aeration. *E. coli* BB4 (*recA*$^+$ *lacI*^{q} Δ*M15*, Tet^r) was grown in Luria-Bertani broth in the presence of 10 μg of tetracycline per ml. Where appropriate, ampicillin was used at a concentration of 100 μg/ml.

Antisera and monoclonal antibodies. Antiserum directed against *L. monocytogenes* was produced by multiple injections of live bacteria into rabbits. Antisera were tested for reactivity and specificity in Western blot (immunoblot) assays (7). The preparation and characterization of monoclonal antibody 111 has been previously described (18, 36, 39).

Preparation of bacteria for Western blotting and surface iodination. *L. monocytogenes* cells were iodinated with Iodogen (Pierce Chemical Co., Rockford, Ill.) by the method of Judd (14) as modified by Shafer and Morse (33). Fresh

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bacterial culture (1 ml) (10^8 to 10^9/ml) was pelleted and suspended in 0.2 ml of cold phosphate-buffered saline (PBS) and added to glass tubes precoated with 100 μl of 100 μg of Iodogen per ml. $Na^{125}I$ (100 μCi) was added, and the mixture was incubated (10 min, 4°C). The reaction was stopped by the addition of cold NaI to a final concentration of 0.25 M. Iodinated bacteria were washed three times with ice-cold PBS to remove unbound isotope. The bacterial pellet was suspended in 50 μl of 2.5-mg/ml lysozyme in PBS and incubated overnight at 37°C. Solubilized proteins (1.5×10^5 cpm/ml) were subjected to electrophoresis on a 10% polyacrylamide gel (19), followed by electroblotting onto nitrocellulose paper and detection by autoradiography.

For Western blot analysis, proteins from unlabeled bacteria were solubilized as described above. After electrophoresis and electroblotting onto nitrocellulose, proteins were subjected to Western blotting procedures with rabbit antisera and monoclonal antibody 111 (hybridoma culture supernatant). Normal (preimmune) rabbit serum was unreactive. Antigen-antibody complexes were detected with ^{125}I-labeled protein A and autoradiography.

Soluble listerial proteins were prepared as described previously (36). The soluble listerial proteins were prepared from freshly expanded and washed live bacteria by sonication (six 5-min cycles, 30 Watts, 4°C), ultracentrifugation (100,000 × *g*, 60 min, 4°C), and fractionation of soluble material by isopycnic gradient centrifugation (37% [wt/wt] cesium chloride, SW50.1 rotor, 150,000 × *g*, 70 h). Material banding at a density of 1.3 g/ml was pooled, dialyzed against PBS, assayed for protein content, and stored at −20°C. Proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting techniques.

Construction and screening of lambda-ZAP library. Genomic DNA from *L. monocytogenes* serotype 1/2a was prepared as described previously (23) except that bacterial cells were suspended in 1 ml of 0.01 M sodium phosphate buffer (pH 7.0) containing 20% sucrose and lysozyme (2.5 mg/ml) and incubated overnight at 37°C before the addition of lysis buffers. A custom DNA library was prepared by using the lambda-ZAP vector (34) from Stratagene, Inc. (La Jolla, Calif.). Fragments of sheared genomic DNA (6 to 10 kilobases [kb]) were ligated to lambda-ZAP arms and packaged into bacteriophage particles. Recombinant phage were plated with *E. coli* BB4, and plaques were screened as follows. After incubation for 6 to 8 h at 37°C, isopropyl-β-D-thiogalactopyranoside-treated nitrocellulose filters were overlaid onto phage plaques for 3 h followed by the addition of a duplicate filter and incubation for an additional 2.5 h. Filters were processed in the same manner as for Western blot assays (7), using rabbit anti-*L. monocytogenes* antiserum at 1:1,000 dilution and monoclonal antibody 111 as undiluted hybridoma culture supernatant.

For screens with synthetic oligonucleotide probes, plaques were transferred as above and processed by standard techniques (2). Initial screens were done with a pool of six oligonucleotide probes derived from the published sequence for *hlyA* (25):

LLO 427–444: GTTGTGGAGAAAAAGAAG
LLO 742–756: GATTATGATGACGAA
LLO 820–855: AGCTTGAATGTAAACTTCGGCGCAATCAGTG
LLO 900–923: CGTGAATGTTAATGAACCTACAAG
LLO 1389–1412: CATCGATCACTCTGGAGGATACGT
LLO 1518–1553: TTTCACATCGTCCATCTATTTGCCAGGTAACGCGAG

Probes were 5′ end labeled with ^{32}P by using T4 polynucleotide kinase. Plaques were transferred to nitrocellulose as described above and processed by standard techniques (2). Hybridization and washing were done by the procedure of Wood et al. (37) to eliminate the effect of base composition on the stringency of hybridization. Positive phage isolates were further screened with the polyclonal rabbit anti-*L. monocytogenes* antiserum as described above.

Preparation of recombinant antigen. Recombinant antigen made by lambda phage clones was prepared as described in detail elsewhere (27, 28). Briefly, recombinant antigen from phage plaques was allowed to diffuse into RPMI 1640 medium plus 1% penicillin-streptomycin for 1 h at 4°C. In assays for T-cell activation with recombinant antigen from phage clones, the concentration of antigen in the preparations tested was controlled at the level of amount of phage plated. Lambda-ZAP phage vector without insert DNA was used as the negative control. Antigen preparations were stored frozen at −70°C until needed.

Insert DNA from lambda-ZAP phage clones was subcloned by excision into pBluescript according to the instructions of the manufacturer (Stratagene). Recombinant phage particles were incubated with *E. coli* and R408 helper phage. The resulting Bluescript plasmid contains the insert DNA and is packaged in F1 phage particles. The plasmid was rescued by transduction of *E. coli* BB4 cells and selected with ampicillin. Recombinant plasmids were expressed in *E. coli* BB4. These bacteria were grown in broth culture to an A_{600} = 0.5 and induced with isopropyl-β-D-thiogalactopyranoside at a final concentration of 10 mM. After 5 h, the cells were pelleted and suspended in 1/20th the original volume of PBS. Cell suspensions were frozen and thawed to lyse the cells, followed by sonication (three times for 10 s, 40 W) to reduce viscosity and sterilization by filtration (28). This preparation was used in T-cell activation assays and stored frozen at −70°C.

DNA manipulations. Standard protocols were used in the preparation of plasmid DNA, digestion with restriction enzymes, ligation, and transformation of competent bacteria (2). The *hlyA* gene was subcloned by digesting plasmid DNA (pT10e) with *Bam*HI based on the published restriction map of *hlyA*-flanking sequences (20), separating the products by agarose electrophoresis, purifying the DNA from the bands of interest, and ligating to *Bam*HI-digested pBluescript.

Sequence analysis of the recombinant plasmid, pT10e, was done with the *hlyA*-specific synthetic oligonucleotide probe 5′-CGATTGGCGTCTTAGGAC-3′ as a primer. The Sequenase Kit, Version 2.0 (United States Biochemical Corp., Cleveland, Ohio) was used according to the instructions of the manufacturer.

Construction of *E. coli* BB4(pT10eM1) frameshift mutant. The plasmid pT10e.2b was digested with *Eco*RI. To create blunt ends, resulting fragments were incubated with 0.125 mM each of the four deoxynucleoside triphosphates and 8 U of Klenow fragment (New England BioLabs, Inc., Beverly, Mass.) at 30°C for 30 min. After extraction with phenol-chloroform and precipitation with ethanol, the DNA was ligated and transformed into *E. coli* BB4. Resulting transformants were screened for loss of *Eco*RI sites and maintenance of *Hin*cII sites. Proper orientation of the fragment was confirmed by sequence analysis with the synthetic oligonucleotide primer LLO 427-444. The parent plasmid, pT10e.2b, and the frameshift mutant, pT10eM1, were also digested with restriction enzymes *AccI*, *ClaI*, and *NcoI* to demonstrate proper orientation of *hlyA*.

T cells and macrophages. Female C3H/Hej mice, 8 to 12 weeks old, were purchased from the Jackson Laboratory (Bar Harbor, Maine). Peritoneal exudate cells were obtained

from mice infected intraperitoneally with 2.5×10^3 viable *L. monocytogenes*, followed by 2.5×10^4 viable *L. monocytogenes* 2 weeks later. One week after the second immunization, the mice received an intraperitoneal injection of 10% proteose peptone (1.5 ml) and were sacrificed 3 days later.

Tissue culture media. Peritoneal lavage was performed with Hanks balanced salt solution containing 0.06% bovine serum albumin, 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer, 2 mM L-glutamine, and heparin (10 U/ml). For cell culture with recombinant antigens, the following medium was used: RPMI 1640 containing 5% fetal calf serum, 10 mM HEPES, 2 mM L-glutamine, 0.075% sodium bicarbonate, 0.5 mM sodium pyruvate, and 50 μg of gentamicin per ml. HT-2 cells (interleukin-2 [IL-2]-dependent cell line) were maintained in Dulbecco modified Eagle medium containing 10% fetal calf serum, penicillin (50 U/ml), and streptomycin (50 μg/ml), and 2 mM L-glutamine supplemented with 10% Rat-T Monoclone (Collaborative Research, Inc., Bedford, Mass.).

Assay for T-cell activation. The ability of recombinant antigens to stimulate *L. monocytogenes*-immune T cells in vitro was measured by the augmented production of IL-2 in culture (40). Dilutions of recombinant antigen were added to 3×10^5 peritoneal exudate cells in a microtiter plate. Heat-killed *L. monocytogenes* was used as the positive control antigen; cell lysates from *E. coli* BB4 and *E. coli* BB4(pBluescript) were used as negative control antigens. After 18 to 24 h, culture supernatants were collected. These supernatants were assayed for the presence of IL-2 with the IL-2-dependent cell line HT-2 (80 μl of supernatant was added to 20 μl of 2×10^5 HT-2 cells per ml). After 24 h, 25 μl of [^{3}H]thymidine (0.025 mCi/ml) was added. [^{3}H]thymidine incorporation was measured 16 to 18 h later and expressed as the mean of duplicate wells.

Effect of LLO on antigen presentation. The ability of macrophages to process and present antigen to T cells was measured by the enhanced production of IL-2 after 18 h in culture as described previously (8). In 96-well microtiter plates, macrophages derived from 2.5×10^5 peritoneal exudate cells (BALB/c mice, *H-2^d*) were plated. Recombinant LLO was added at concentrations indicated in the figure legends together with the antigen, chicken ovalbumin (OVA, 100 μg per well), and the plates were incubated for 2 h at 37°C. Macrophages were washed and fixed with 0.05% glutaraldehyde in PBS for 2 min, followed by the addition of glycine (2.19%). Cells were washed four times with cell culture medium. Responder T cells (T-cell hybridoma DO11.10 [24] which reacts with chicken OVA/*H-2^d*) were added at 1×10^5 to 2×10^5 cells per well. After 18 h, a portion of each supernatant was collected. Supernatants were added to HT-2 cells, an IL-2-responsive cell line, and assayed as described above.

Determination of hemolytic activity. LLO from *L. monocytogenes* was prepared as follows. Bacteria were grown for 18 to 24 h at 37°C with constant aeration in brain heart infusion medium supplemented with 1% glucose. Cells were removed by centrifugation (1,500 × *g* for 30 min at 4°C), and the resulting supernatant was sterilized by filtration. Phenylmethylsulfonyl fluoride and EDTA were added to final concentrations of 1 and 10 mM, respectively. Proteins in the solution were precipitated by the addition of solid ammonium sulfate (53 g/100 ml) with stirring for 8 to 12 h at 4°C. The resulting precipitate was collected by centrifugation, suspended in PBS (1/100th of the original volume), and dialyzed against PBS containing 4 mM EDTA (18 to 24 h at 4°C, with at least three buffer changes). These preparations were stored at 4°C.

Bacterial cell lysates and culture supernatants were tested for hemolytic activity in a microtiter assay. Samples of 90 μl of PBS (negative control), distilled H_2O (positive control, total lysis), or LLO preparations were added to individual wells of a microdilution plate, followed by the addition of 10 μl of 100 mM dithiothreitol. The plates were incubated for 10 min at 37°C, and sheep erythrocytes were added to a final concentration of 1%. The plates were incubated an additional 30 min at 37°C. Erythrocytes were pelleted by centrifugation, and the wells were scored for hemolysis. Supernatants were collected with a multichannel pipetter and transferred to clean microdilution plates for spectrophotometric analysis at 414/415 nm (Titertek Multiskan plate reader; Flow Laboratories, Inc., McLean, Va.). Total lysis was determined by the addition of 90 μl of distilled H_2O to the test wells. One hemolytic unit is defined as the minimum amount of lysin to induce 50% lysis of sheep erythrocytes and is expressed as the reciprocal of the dilution.

RESULTS AND DISCUSSION

Definition of antibodies used to screen for listerial antigens. The proteins recognized by the polyclonal rabbit anti-*L. monocytogenes* antiserum and those exposed on the surface of live *L. monocytogenes* cells were characterized as follows. Surface proteins were selectively labeled with ^{125}I by the method of Judd (14) as modified by Shafer and Morse (33), electrophoresed on 10% polyacrylamide gels, and compared with Western blot profiles of solubilized listerial proteins by using the antiserum. To permit direct comparisons with the Western blot reactivity patterns, iodinated surface proteins were separated by SDS-PAGE and transferred to nitrocellulose together with material from unlabeled bacteria prepared in parallel. The portion of the blot with unlabeled material was probed with rabbit antiserum, and then the nitrocellulose sheet was reconstructed for autoradiography. At least four bands which were detected with the *L. monocytogenes*-specific rabbit antiserum (Fig. 1, lane B) were also detected in the autoradiogram of iodinated listerial proteins (lane A). These bands correspond to proteins with apparent molecular masses of 77, 62, 45, and 39 kilodaltons (lane B). The correspondence of surface iodinated proteins with bands detected by Western blotting with the rabbit anti-*L. monocytogenes* antiserum suggests that the major antigens of *L. monocytogenes* are cell surface proteins. The surface location of these proteins was supported by the ability of intact live *L. monocytogenes* to bind to and fully deplete the antiserum of reactivity to these bands (data not shown). The specificity of these reactions is illustrated by the observation that antigens associated with *E. coli* were unreactive with the anti-*L. monocytogenes* serum (lane C).

Two of the surface-exposed proteins (62 and 39 kilodaltons) which bound to the rabbit anti-*L. monocytogenes* antiserum were also recognized by a murine hybridoma-derived monoclonal antibody 111. Western blotting experiments with this monoclonal antibody identified proteins with apparent molecular masses of 62, 59, and 39 kilodaltons in solubilized *L. monocytogenes* (Fig. 2). An identical binding pattern was seen when SDS-PAGE was performed under reducing conditions (data not shown). This multiple banding pattern in Western blots with monoclonal antibody 111 may be the result of fragmentation of the antigen during preparation, a relationship among these proteins by some biosyn-

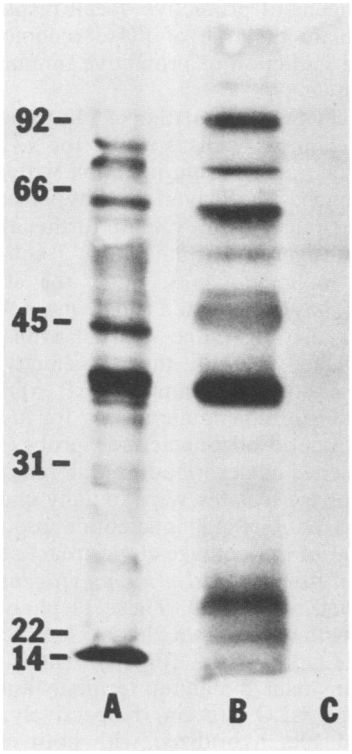


FIG. 1. Comparison of ^{125}I-labeled surface-exposed proteins of *Listeria monocytogenes* by Western blot analysis. Lane A, Autoradiograph of ^{125}I-labeled surface proteins from *L. monocytogenes*. Lanes B and C, Western blot with solubilized *L. monocytogenes* and *E. coli*, respectively. Antigens were detected with a 1:1,000 dilution of rabbit anti-*L. monocytogenes* antiserum.

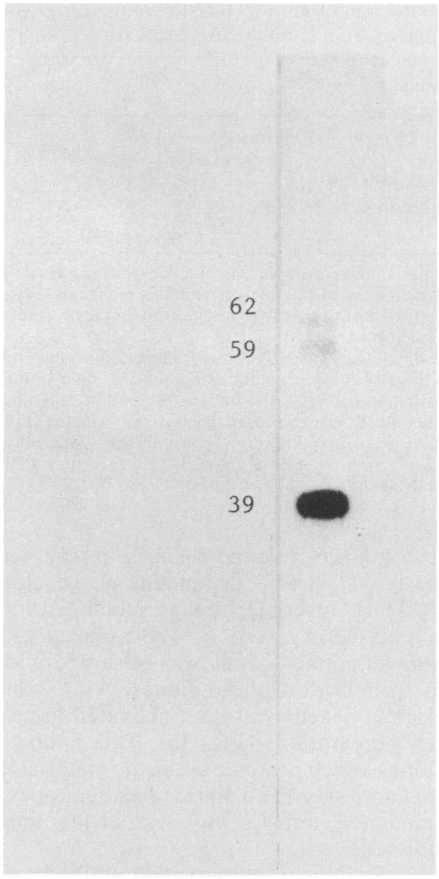


FIG. 2. SDS-PAGE and Western blot analysis of solubilized live *L. monocytogenes*. Live *L. monocytogenes* (10^9 freshly expanded and washed live bacteria) were incubated overnight at 37°C in PBS containing 2.5 mg of lysozyme per ml, followed by heating (100°C, 2 min) in SDS-PAGE sample buffer (equal volume). After removal of particulate matter by centrifugation, 100 μl of solubilized material was applied to the gel. Monoclonal antibody 111 was used in the Western blot analysis at a 1:2 dilution. Numbers on left show molecular size in kilodaltons.

thetic or catabolic pathway, or the simple sharing of antigenic epitopes among the different proteins.

Strategy for cloning and isolation of recombinant antigens with antibodies. Since protection against *L. monocytogenes* is mediated by antigen-specific T cells, our ultimate objective was to identify listerial antigens important in eliciting this response. Because there is no convenient method to screen large numbers of recombinant phage directly with T cells, a two-stage screening protocol was used. The phage library was first screened with a polyclonal rabbit anti-*L. monocytogenes* antiserum and *L. monocytogenes*-specific monoclonal antibody 111. In each case, positive isolates were further evaluated for reactivity with *L. monocytogenes*-immune T cells. Consequently, we were able to screen large numbers of phage plaques in colony blot assays using antibody reagents followed by the more difficult and time-consuming assay with immune T cells. A basic assumption inherent to this strategy was that both antibody (B cell) and T-cell epitopes would be present on a given listerial antigen, and thus the same molecule would be detected with either reagent. One disadvantage of this system is that clones expressing molecules that bear only T-cell epitopes would not be detected.

Isolation of recombinant antigens with antibodies. According to the strategy as described above, recombinant antigen from lambda-ZAP phage clones was tested for reactivity first with antibody and then with *L. monocytogenes*-immune T cells. Of the approximately 120,000 phage screened, 69 plaque-purified phage isolates gave consistent reactivity with the rabbit anti-*L. monocytogenes* antiserum and were chosen for further study. When antigenic material from phage plaques was tested for T-cell antigenicity, 19 of the 69 isolates caused significant activation of *L. monocytogenes*-immune T cells (Table 1). The observation that T-cell reactivity was noted in only 27% of the antibody-reactive isolates may indicate that there is only partial overlap in the repertoire of T-cell and B-cell receptors with respect to listerial antigens. It is also possible, however, that screening with antibody probes is more sensitive than detecting reactivity with polyclonal populations of *L. monocytogenes*-immune T cells.

Antigen from one of these isolates, lambda-ZAP clone B7.2, induced very strong and consistent activation of *L. monocytogenes*-immune T cells. DNA from phage clone B7.2 was subcloned into the plasmid vector pBluescript and expressed in *E. coli* BB4. Antigen prepared from the lysate of these bacterial cells was tested for ability to stimulate *L. monocytogenes*-immune T cells in vitro. T cells responded strongly to this antigen preparation in a dose-dependent manner (Fig. 3a). Negligible responses were observed with T cells obtained from control mice (Fig. 3b). Thus, T cells specific for antigenic epitopes represented by clone B7.2 were generated during infection with *L. monocytogenes*.

TABLE 1. Detection of murine T-cell-reactive *L. monocytogenes* antigens from lambda-ZAP expression library

Screening method[a]	Positive clones	T-cell-reactive clones[b]
a. Rabbit anti-*L. monocytogenes* antiserum	69	19
b. Monoclonal antibody 111	2	2
c. *hlyA*-specific oligonucleotide probes	28	7

[a] Recombinant lambda-ZAP phage plaques were screened by Western blotting techniques (a and b) and Southern blotting techniques (c) as described in Materials and Methods. In each case, positive clones were chosen and evaluated for T-cell reactivity.

[b] Recombinant antigen was allowed to diffuse from phage plaques into RPMI 1640 medium plus 1% penicillin-streptomycin and cocultured with *L. monocytogenes*-immune T cells and antigen-presenting cells. Production of IL-2 by responder T cells was evaluated in a bioassay with the IL-2-dependent cell line HT-2. Antigen preparations which induced statistically significant activation of *L. monocytogenes*-immune T cells are indicated as T-cell-reactive clones (Student's t test, $P < 0.05$).

Upon screening the genomic *Listeria* library with monoclonal antibody 111, two independent phage clones were obtained (Table 1). Insert DNA was subcloned into pBluescript and expressed in *E. coli* BB4. Strong activation of *L. monocytogenes*-immune T cells was observed with antigen preparations from both of these clones, A111.2 and F111.2 (Fig. 3a). Normal T cells were not activated in response to these antigen preparations (Fig. 3b). This finding suggests that these clones which produce antigenic material that binds to monoclonal antibody 111 also represent epitopes to which the T-cell-mediated anti-*L. monocytogenes* immune response is directed.

Among the recombinant clones with antibody reactivity, antigen produced by B7.2, A111.2, and F111.2 induced strong activation of *L. monocytogenes*-immune T cells (Fig. 3). Because of the consistent potency of these antigen preparations, we believe these to be candidates for important target antigens of protective T-cell responses. Experiments to evaluate the role of these recombinant listerial antigens in the induction of protective immunity are under way.

Strategy for cloning and isolation of LLO-producing recombinant clones. Since T cells specific for LLO have been shown to be generated during infection with *L. monocytogenes* as evidenced by delayed-type hypersensitivity reactions (4), it was relevant to evaluate further the role of this protein as an antigen important in the T-cell-mediated immunity to *L. monocytogenes*. *hlyA*, the structural gene encoding LLO, has been cloned and sequenced by Mengaud et al. (25). Because this clone was not available to us and because we wished to study the antigenicity of LLO, we chose to clone *hlyA* into the lambda-ZAP expression vector. To screen the *Listeria* genomic library for *hlyA*, we used a pool of six synthetic oligonucleotide probes based on the published sequence as described in Materials and Methods. Twenty-eight phage isolates were initially chosen based on binding to this pool of oligonucleotide probes (Table 1). Antigen from 11 of these phage clones reacted strongly with the polyclonal rabbit anti-*L. monocytogenes* antiserum, which also reacts with LLO. These 11 phage clones were further tested with each of two oligonucleotide probes, LLO 427-444 and LLO 1518-1553 (Fig. 4). These probes correspond to regions near the amino terminus and the carboxy terminus of the LLO protein, respectively. Two of the clones, 2c and 10e, hybridized with both oligonucleotide probes. The remainder of the clones hybridized to LLO 1518-1553 with various degrees of intensity or to neither of the probes (Fig. 4).

To evaluate the hemolytic activity of recombinant LLO, insert DNA from lambda-ZAP clones, 2c and 10e, was subcloned into the pBluescript vector and expressed in *E. coli* BB4. The resulting subclones, *E. coli* BB4(pT2c) and *E. coli* BB4(pT10e), were streaked onto blood agar plates to test for hemolysis. Wild-type *L. monocytogenes* and *E. coli*

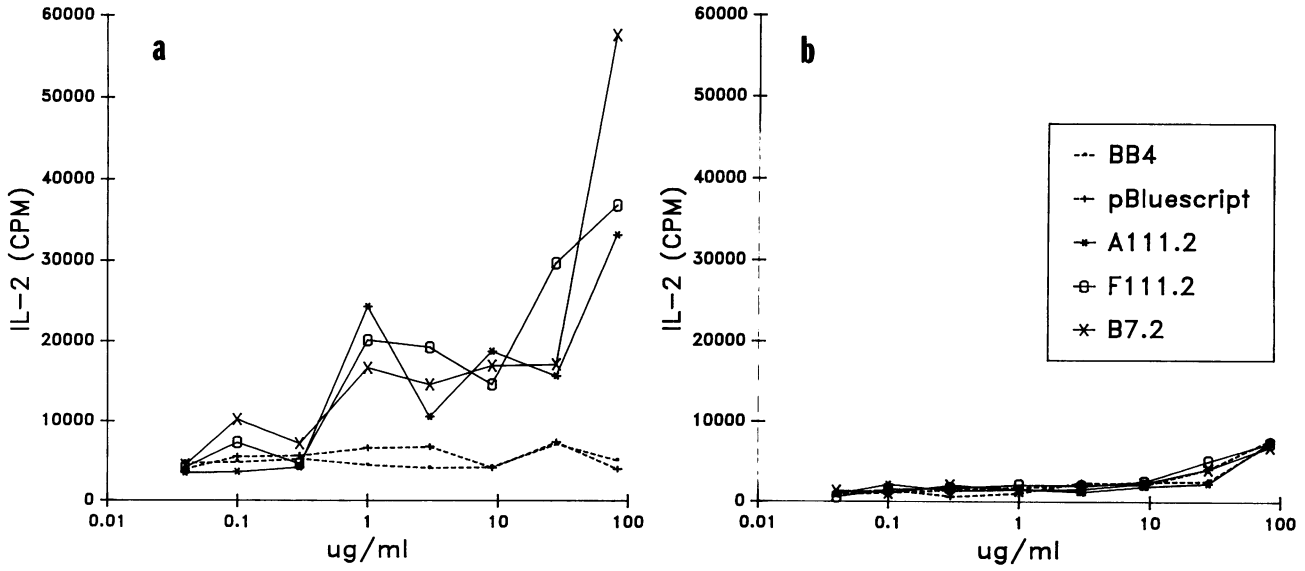


FIG. 3. T-cell antigenicity of cloned listerial antigens. (a) Bacterial cell lysates from the following clones were prepared as described in Materials and Methods: *E. coli* BB4 host cell, *E. coli* BB4(pBluescript), A111.2 and F111.2 (selected by binding to monoclonal antibody 111), and B7.2 (selected by binding to polyclonal rabbit anti-*L. monocytogenes* antiserum). Antigen was cocultured with *L. monocytogenes*-immune T cells and antigen-presenting cells, and supernatants were evaluated for IL-2. (b) Bacterial cell lysates were cocultured with T cells and antigen-presenting cells taken from unimmunized mice, and IL-2 production was evaluated.

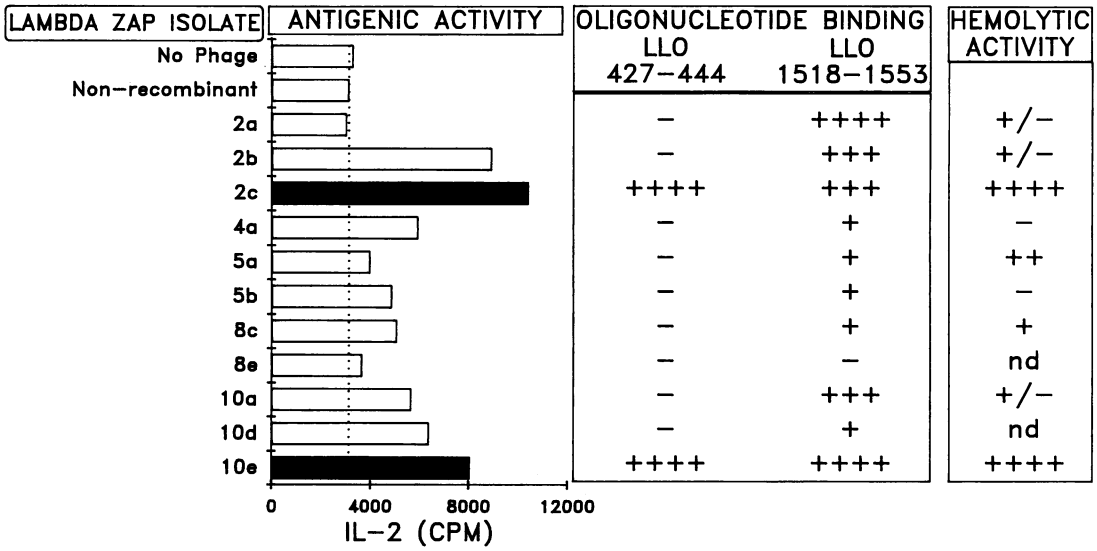


FIG. 4. Activation of *L. monocytogenes*-immune T cells with recombinant antigen selected by LLO-oligonucleotide binding. Antigenic activity, Antigenic material from recombinant phage plaques was allowed to diffuse into RPMI 1640 medium plus 1% penicillin-streptomycin and cocultured with *L. monocytogenes*-immune T cells and antigen-presenting cells. IL-2 production was evaluated as previously described. Oligonucleotide binding, Scores refer to subjective evaluation of intensity of binding signals on autoradiographs. Hemolytic activity, scores refer to subjective evaluation of hemolytic plaque formation by recombinant bacterial colonies on blood agar plates containing 100 μg of ampicillin per ml.

containing the recombinant plasmids pT2c and pT10e produced strong zones of beta-hemolysis on blood agar plates. In contrast, no hemolysis was evident on plates streaked with *E. coli* BB4 host cells or host cells containing pBluescript (Fig. 5). For the clone *E. coli* BB4(pT10e), hemolysis was observed in the presence and the absence of isopropyl-β-D-thiogalactopyranoside, indicating that expression of the cloned protein was independent of the *lac* promoter (data not shown).

Although both clones, pT2c and pT10e, produced zones of hemolysis on blood agar plates (Fig. 5), no detectable hemolytic activity was found in culture supernatants of either clone (Table 2). Hemolytic activity of both clones was exclusively associated with the cell lysate. The finding of hemolysis on blood agar plates without evidence for export in broth culture may be explained by the possible autolysis of bacterial cells in colonies, resulting in the liberation of LLO. Alternatively, the local concentration of LLO associated with colonies on blood agar plates may be sufficient to create zones of hemolysis. In addition, it is possible that recombinant LLO produced by *E. coli* is extremely labile or may be rapidly inactivated by some component of the *E. coli* culture supernatant.

Greater hemolytic activity was consistently detected in cell lysates from *E. coli* BB4(pT10e) (Table 2), although the hemolytic activity associated with bacterial cell lysates of this clone was far less than that found in culture supernatants of wild-type *L. monocytogenes* (13,850 hemolytic units [HU]/ml ± 3,155 [standard deviation], $n = 5$ experiments). It appears that the regulation of expression of this gene differs in gram-negative organisms compared with the gram-positive *L. monocytogenes*.

The phage clones, 2c and 10e, were also noted to express antigenic material that consistently activated *L. monocytogenes*-immune T cells in vitro (Fig. 4), a property which will be discussed in greater detail below. Because of this antigenic activity, the oligonucleotide binding profile, and the observed hemolytic activity, these clones were chosen for more detailed analysis.

To confirm the presence of the *hlyA* in pT10e, we performed sequence analysis of the insert DNA in pT10e with the *hlyA*-specific oligonucleotide primer as described in Materials and Methods. The insert size of listerial DNA in the recombinant plasmid, pT10e, was 6.9 kb. The sequence obtained (base pairs 24 to 183) confirmed the presence of the *hlyA* gene and the presence of its intact wild-type promoter (data not shown).

hlyA was subcloned into the pBluescript vector such that the insert size favored the exclusive expression of recombinant LLO. This was accomplished by cloning the *Bam*HI fragment containing *hlyA* into pBluescript at the *Bam*HI site in the polylinker of this vector (20). Digestion of pT10e with *Bam*HI yielded two fragments large enough to contain *hlyA*: one fragment of 5.0 kb and another fragment of 3.2 kb. Each of these fragments was ligated to pBluescript at the *Bam*HI site in the polylinker of this vector and used to transform *E. coli*. The resulting ampicillin-resistant subclones were streaked on blood agar plates to evaluate functional hemolytic activity. One subclone, designated pT10e.2b, that resulted from the subcloning of the 3.2-kb fragment produced strong zones of hemolysis compared with *E. coli* BB4(pBluescript). As with the parent clone, *E. coli*(pT10e), described above, strong hemolytic activity was detected in the cell lysates of *E. coli*(pT10e.2b), with negligible activity found in the culture supernatants (Table 2, experiment 2). Like wild-type LLO, recombinant LLO was irreversibly inactivated by heat and cholesterol (Table 2, experiments 1 and 3). The presence of *hlyA* in the subclone BB4(pT10e.2b) was confirmed by restriction map analysis and Southern hybridization with an *hlyA*-specific oligonucleotide probe, LLO 1518-1553.

Activation of *L. monocytogenes*-immune T cells by recombinant LLO. In initial evaluations, antigenic material from phage clones 2c and 10e significantly stimulated *L. monocy-*

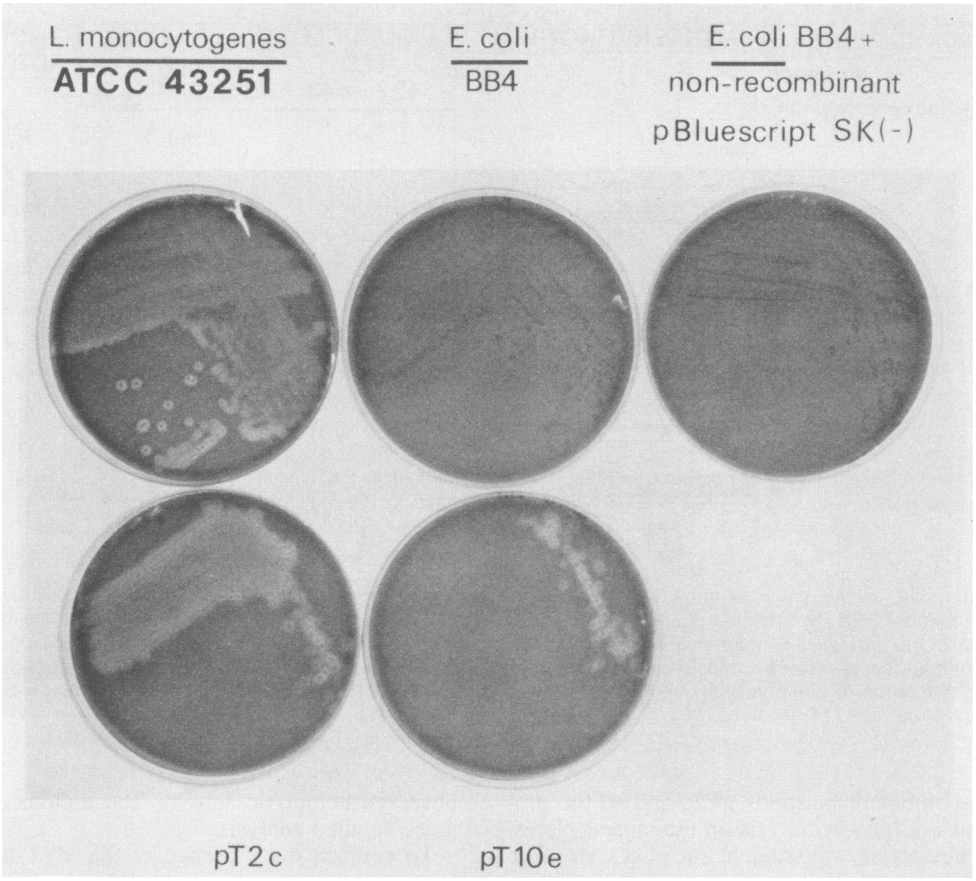
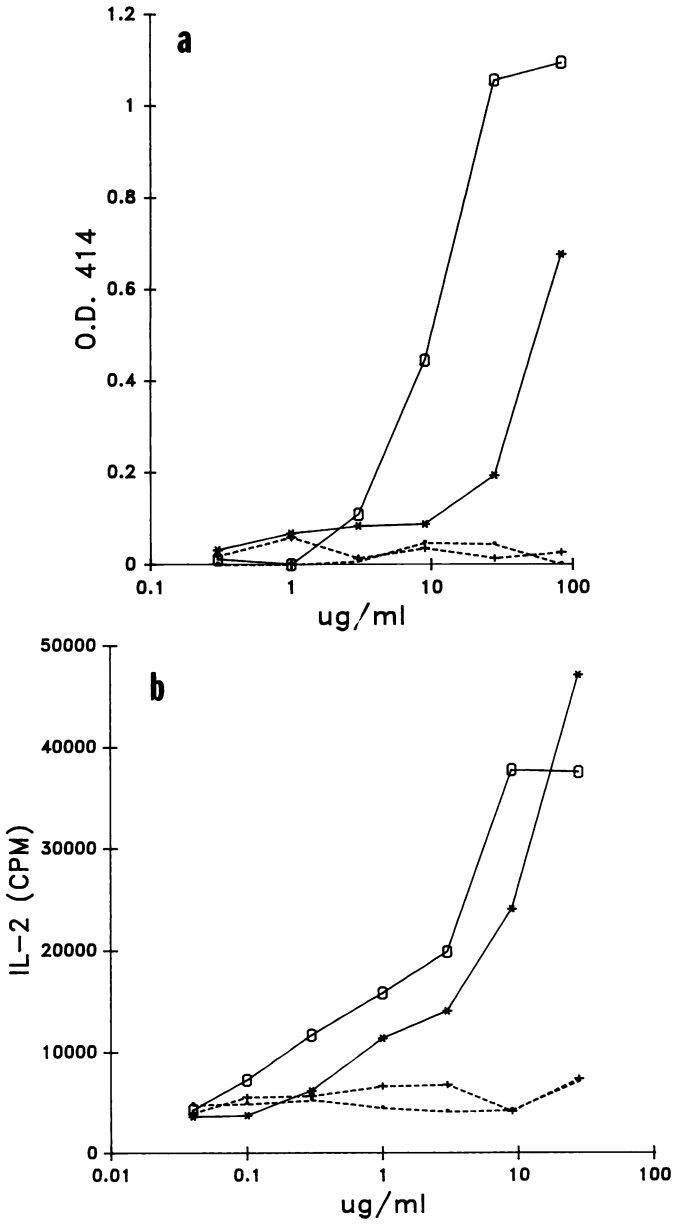


FIG. 5. Hemolysis on blood agar plates. *L. monocytogenes* was streaked onto blood agar plates and incubated for 48 h at 37°C. *E. coli* BB4, *E. coli* BB4(pBluescript), *E. coli* BB4(pT2c), and *E. coli* BB4(pT10e) were streaked on blood agar plates containing 100 μg of ampicillin per ml and 10 mM isopropyl-β-D-thiogalactopyranoside incubated for 24 h at 37°C. Appearance of hemolytic plaques as the result of lysis by LLO produced by each test strain was evaluated after 24 and 48 h for *E. coli* clones and *L. monocytogenes* (ATCC 43251), respectively.

TABLE 2. Determination of hemolytic activity

Expt	Hemolytic activity (HU/ml)[a]					
	Culture supernatant	Cell lysate	Heat inactivated[b]		Minus cholesterol	Plus cholesterol[c]
			Supernatant	Lysate		
1						
L. monocytogenes	18,410	1,780	<1	<1		
E. coli BB4	<1	<1	<1	<1		
E. coli BB4(pBluescript)	<1	<1	<1	<1		
E. coli BB4(pT2c)	<1	80	<1	<1		
E. coli BB4(pT10e)	<1	630	<1	<1		
2						
E. coli BB4(pBluescript)	<1	<1				
E. coli BB4(pT10e.2b)	<1	373				
3						
L. monocytogenes (supernatant)					10,740	3
E. coli BB4(pBluescript) (lysate)					<1	<1
E. coli BB4(pT2c) (lysate)					156	<1
E. coli BB4(pT10e) (lysate)					460	<1

[a] Cell lysates or culture supernatants were tested for hemolytic activity as described in Materials and Methods.
[b] Cell lysates or culture supernatants were heated for 30 min at 56°C before testing.
[c] Cell lysates or culture supernatants were tested for hemolytic activity in the presence of 100 μg of cholesterol per ml.



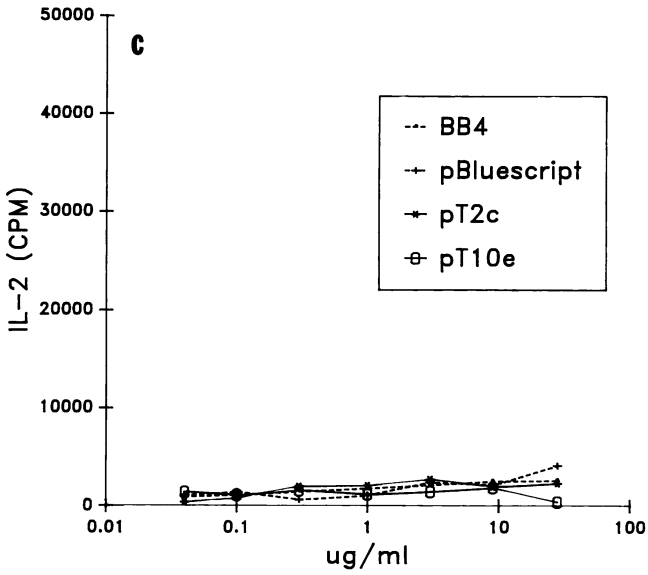


FIG. 6. (a) Hemolytic activity in each preparation of recombinant antigen was determined as described in Materials and Methods. O. D. 414; Optical density at 414 nm. (b) Activation of *L. monocytogenes*-immune T cells by recombinant LLO antigen. Bacterial cell lysates of *E. coli* BB4 and recombinant clones were used as the antigen and cocultured with *L. monocytogenes*-immune T cells and antigen-presenting cells. Culture supernatants were evaluated for IL-2 by a bioassay as described in Materials and Methods. (c) Activation of normal T cells by recombinant LLO antigen. Bacterial cell lysates were cocultured with T cells taken from nonimmunized mice. Culture supernatants were evaluated for IL-2.

togenes-immune T cells (Fig. 4). Insert DNA from these recombinant phage was subcloned into pBluescript and expressed in *E. coli* BB4. Antigen prepared from the cell lysates of these bacteria was tested for reactivity with *L. monocytogenes*-immune T cells in vitro. Bacterial cell lysates from *E. coli* BB4(pT2c) and *E. coli* BB4(pT10e) strongly stimulated *L. monocytogenes*-immune T cells in a dose-dependent manner, in contrast to the lack of activation induced by *E. coli* BB4 alone or *E. coli* BB4(pBluescript) at equal protein concentration (Fig. 6b). Antigenic activity was detectable at concentrations lower than those required for detection of hemolytic actvity (Fig. 6a and b). None of the antigens tested induced activation of T cells taken from nonimmunized mice (Fig. 6c). These data establish that T cells specific for LLO are induced by infection with *L. monocytogenes* in mice and that only *E. coli* lysates which contain recombinant LLO antigen stimulated *L. monocytogenes*-immune murine T cells.

The results from several experiments with different batch preparations of recombinant LLO antigen are shown in Table 3. Data from the activation of *L. monocytogenes*-immune T cells in response to antigenic material allowed to diffuse from recombinant phage plaques are summarized in the top portion of Table 3. Data from batches 6 to 11 refer to activation of *L. monocytogenes*-immune T cells in response to antigen in the form of bacterial cell lysates of recombinant clones. Although the absolute numbers vary from batch to batch, recombinant LLO antigen consistently stimulated *L. monocytogenes*-immune T cells when compared with the negative control antigens from *E. coli* BB4 or *E. coli* BB4(pBluescript). Also shown in this table is the antigenic activity of recombinant LLO produced by *E. coli* BB4(pT10e.2b). Expression of recombinant LLO in strains of *E. coli* other than BB4 (i.e., MG/1655) resulted in significant activation of *L. monocytogenes*-immune T cells, whereas a negligible response was seen with preparations from bacteria containing the nonrecombinant plasmid or no plasmid at all (data not shown). Thus, it is unlikely that a contaminant in recombinant antigen preparations from *E. coli* contributes to the activation of *L. monocytogenes*-immune T cells in response to recombinant LLO.

Since two additional open reading frames have recently been identified in close proximity to the coding sequence of *hlyA* (26), it was necessary to prove that the activation of *L. monocytogenes*-immune T cells was induced by recombinant LLO expressed in recombinant *E. coli* and not by a protein encoded by the other reading frames. A frameshift mutation was generated in the cloned *hlyA* gene contained in the plasmid pT10e.2b in a manner that left the other two reading frames undisturbed (see Materials and Methods).

TABLE 3. Activation of *L. monocytogenes*-immune T cells by recombinant LLO antigen: batch-to-batch comparison[a]

Assay date	Batch	Medium alone	Negative control		Heat-killed *L. monocytogenes*	pT2c	pT10e	pT10e.2b
			BB4	pBluescript				
Antigen from phage lysates								
1/23	1	2.2 ± 0.4			50.1 ± 2.7		5.0 ± 0.4	
1/31	2	1.2 ± 0.1			70.1 ± 20.9	9.3 ± 7.8	3.0 ± 0.1	
2/9	3	2.4 ± 0.6			18.1 ± 2.3	4.3 ± 0.7	4.6 ± 0.5	
2/25	4	3.1 ± 0.3			106.8 ± 3.8	9.8 ± 0.0	7.1 ± 2.4	
3/5	5[b]	2.3 ± 0.2			99.0 ± 8.5	10.7 ± 0.8	19.9 ± 1.8	
Antigen from bacterial cell lysates								
3/11	6[b]	0.2 ± 0.0		0.6 ± 0.0	71.4 ± 0.7	42.1 ± 0.8	130.2 ± 15.5	
4/30	7[b]	1.7 ± 0.5	4.2 ± 0.0	4.1 ± 0.4	63.3 ± 1.1	24.0 ± 5.6	37.7 ± 1.9	
7/17	8[b]	2.5 ± 0.3	3.3 ± 0.9	3.2 ± 0.1	137.6 ± 6.6	15.4 ± 6.9	36.0 ± 1.5	
7/18	9[b]	0.3 ± 0.0		0.8 ± 0.1	95.6 ± 0.6	5.5 ± 1.2	6.2 ± 1.0	
8/4	10[b]	0.3 ± 0.0	0.4 ± 0.0	1.0 ± 0.1	9.0 ± 0.2	2.0 ± 0.2	3.6 ± 0.0	
11/4	11[b]			3.2 ± 2.1				9.7 ± 0.6
				2.7 ± 0.3				16.9 ± 3.2[c]

[a] All antigens are expressed in *E. coli* BB4. Antigens from phage lysates were controlled at the level of phage plated per well (10 μl of approximately 10^8/ml). Antigens from bacterial cell lysates were used at 3 to 10 μg/ml; equal amounts of protein were used within each experiment. The positive control antigen, heat-killed *L. monocytogenes*, was used at 10^6 bacteria per ml. Data are expressed as 10^3 cpm of IL-2 ± standard deviation.

[b] In batches 5 to 11, antigen preparations from *E. coli*(pT10e) were heated for 30 min at 56°C before the T-cell activation assay.

[c] Recombinant antigen tested was a 50% ammonium sulfate precipitate fraction of bacterial cell lysate.

Hemolytic activity and antigenic activity of bacterial cell lysate preparations of the frameshift mutant *E. coli* BB4(pT10eM1) and the parent clone *E. coli* BB4(pT10e.2b) were compared. Negligible hemolytic activity (2 HU/ml) was associated with the bacterial cell lysate of *E. coli* BB4(pT10eM1). This activity was comparable to that found in the lysate of the negative control, *E. coli* BB4(pBluescript) (5 HU/ml). In contrast, 128 and 219 HU/ml were detected in the lysate of *E. coli* BB4(pT10e.2b) and the positive control, LLO in brain heart infusion broth, respectively. Western blot analysis of bacterial cell lysate proteins from *E. coli* BB4(pT10eM1) failed to detect the 58-kilodalton band seen in wild-type LLO-containing supernatant preparations and bacterial cell lysate preparations from recombinant LLO clone *E. coli* BB4(pT10e.2b) (data not shown).

The same bacterial cell lysate preparations were tested for antigenic activity with *L. monocytogenes*-immune T cells. The response of *L. monocytogenes*-immune T cells to preparations from *E. coli* BB4(pT10eM1) were comparable to those of the negative control, *E. coli* BB4(pBluescript) (Fig. 7). These data demonstrate that recombinant LLO is the antigen to which *L. monocytogenes*-immune T cells respond in bacterial cell lysates of *E. coli* BB4(pT10e.2b) and further strengthen the finding that LLO is a strong antigen to which

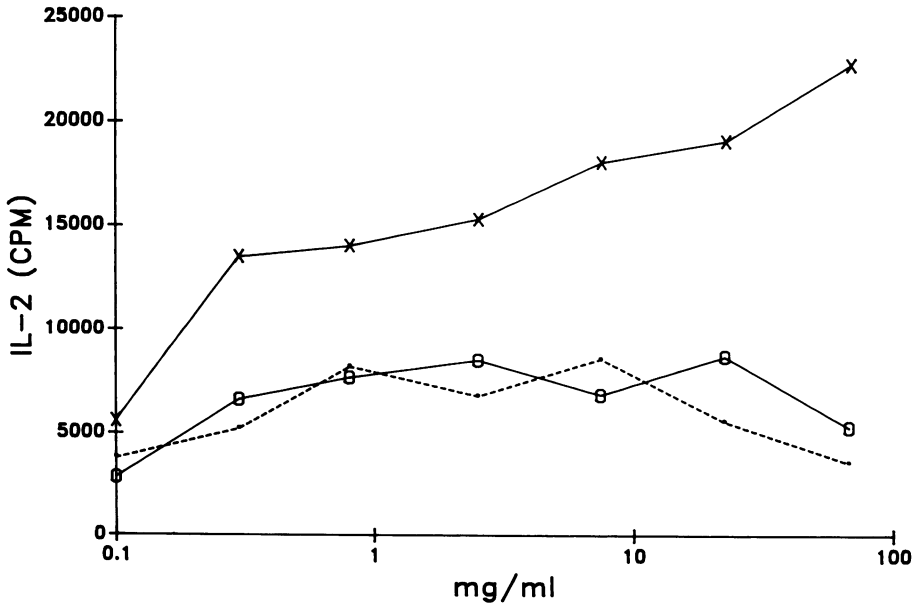


FIG. 7. Activation of *L. monocytogenes*-immune T cells in response to recombinant LLO and mutated recombinant LLO. Antigen in the form of bacterial cell lysates was incubated with *L. monocytogenes*-immune T cells and antigen-presenting cells. Culture supernatants were evaluated for IL-2 by a bioassay described in Materials and Methods. Symbols: ---, BB4(pBluescript); ×, BB4(p10e.2b); ○, BB4(pT10eM1).

the murine immune response to *L. monocytogenes* is directed and that this response is specific for recombinant LLO in *E. coli* lysates.

Development of antilisterial immunity is dependent on immunization with viable, virulent organisms (1, 4, 5, 38a). Bacteria which do not produce LLO are also unable to induce protective immunity against this pathogen. The role of LLO as an antigen was demonstrated by Berche et al. (4) in studies describing delayed-type hypersensitivity reactions in mouse footpads induced by purified wild-type LLO after immunization with sublethal doses of *L. monocytogenes*. Our results extend these findings by establishing the immunogenicity of recombinant LLO in vitro. The antigenic potency of LLO suggests that this antigen is an important target of the T-cell-mediated immune response against pathogenic *L. monocytogenes* (Fig. 6 and 7; Table 3). Furthermore, in preliminary experiments, we have found that immunization of mice with *E. coli* expressing recombinant LLO resulted in the generation of T cells which react with LLO in vitro (data not shown). The vaccine potential of these recombinant antigen preparations is currently being evaluated.

Since viable, LLO-producing *L. monocytogenes* cells are necessary for pathogenesis (9, 13, 15, 30) and for the generation of protective immunity against *L. monocytogenes* (4, 5), LLO may be a signal to the host of an active infection. Because dead or non-LLO-producing *L. monocytogenes* cells do not cause disease, to mount an immune response against these organisms would be unnecessary. These observations suggest the evolution of a successful immunological strategy to combat facultative intracellular pathogens by preferential recognition of the products of live microbes (35, 38). Alternatively, since non-LLO-producing organisms are rapidly cleared, perhaps precluding the development of immunity, the secretion of LLO may simply prolong survival of these organisms in the host, thus potentiating the exposure of the pathogen to the immune system and allowing the development of a productive immune response. Another possibility is that LLO acts as an adjuvant, perhaps by increasing the expression of Ia. Experimental evidence for this hypothesis is the observation that expression of class II major histocompatibility complex gene products was upregulated by infection with LLO-producing *L. monocytogenes* but not by infection with non-LLO-producing organisms (N. E. Marshall and H. K. Ziegler, submitted for publication).

Effect of recombinant LLO on presentation of antigen by macrophages to T cells. We observed that recombinant LLO antigen induced activation of *L. monocytogenes*-immune T cells in vitro in a dose-dependent manner (Fig. 6b). However, the observed antigenic activity of recombinant LLO was decreased at concentrations of LLO antigen higher than those shown in Fig. 6b. It has previously been shown that preincubation of macrophages with LLO-producing but not LLO-negative *L. monocytogenes* impaired the ability of macrophages to present antigen to T cells (8). Thus, these data suggested that the membrane-active properties of recombinant LLO were affecting normal T cell-macrophage interactions in *in vitro* assays. The presentation of listerial antigens (heat-killed *L. monocytogenes*) to *L. monocytogenes*-immune T cells was unaffected by the addition of lysates from nonrecombinant *E. coli* or heat-inactivated wild-type LLO (data not shown). Heating of the antigenic material derived from recombinant LLO clones to destroy hemolytic activity (Table 2) resulted in stronger activation of *L. monocytogenes*-immune T cells against this preparation compared with the unheated preparation (Table 4). These data suggest that inactivation of the membrane-active cytolytic properties of LLO allows optimal T-cell activation in response to this antigen.

TABLE 4. Effect of heat inactivation of hemolytic activity on activation of *L. monocytogenes*-immune T cells by LLO antigen

Antigen in vitro	IL-2 (cpm [10^3])[a]	
	Expt 1	Expt 2
No antigen	2.0 ± 0.6	1.7 ± 0.4
E. coli BB4(pBluescript) (lysate)[b]	0.2 ± 0.1	3.9 ± 0.8
E. coli BB4(pT10e) (lysate)[b]	0.7 ± 0.2	6.6 ± 0.3
Heat-inactivated *E. coli*(pT10e) (lysate)[b]	19.0 ± 1.8	66.4 ± 2.5
Heat-killed *L. monocytogenes*[c]	98.0 ± 8.5	63.3 ± 1.0
LLO[d]	86.0 ± 2.2	58.3 ± 0.1
LLO 215-234[e]	ND[f]	26.0 ± 4.2

[a] Each value represents average counts per minute of duplicate wells ± standard deviation.

[b] Bacterial cell lysates were processed as described in Materials and Methods. Antigen was added to *L. monocytogenes*-immune T cells at a 1:4 dilution or approximately 80 μg/ml.

[c] Used at a concentration of 10^6/ml.

[d] LLO refers to partially purified LLO from bacterial culture supernatants of hemolytic *L. monocytogenes* used at 10 μg/ml.

[e] LLO 215-234 (SQLIAKFGTAFKAVNNSLNV), a synthetic peptide containing amino acids 215 to 234 of LLO, was added to T cells at 10 μg/ml. This antigenic peptide represents a dominant epitope of LLO (S. A. Safley and H. K. Ziegler, unpublished data; 38a).

[f] ND, Not done.

In light of these data and the observations that LLO-expressing *L. monocytogenes* bacteria have been shown to inhibit antigen presentation by macrophages (8), we examined the effect of recombinant LLO on the presentation of the antigen OVA to OVA-specific T-cell hybridomas (DO11.10). Antigen-presenting cells were preincubated with soluble material from the indicated sources together with the antigen, OVA, prior to the addition of OVA-specific T cells. Consequently, it is only the T-cell response to the presentation of the antigen, OVA, which is measured in this system. Recombinant LLO was a potent inhibitor of antigen presentation in this system (Fig. 8). In fact, recombinant LLO antigen produced by *E. coli* BB4(pT10e) inhibited antigen presentation as well as the wild-type LLO. Other researchers (21) have shown that heat-killed *L. monocytogenes* inhibited the presentation of lysozyme antigen. However, since LLO is an exported protein in wild-type *L. monocytogenes*, LLO would not be present in washed, heat-killed bacteria and thus would not be operative in this system.

In our assay system, we have also observed inhibition of OVA presentation with heat-killed *L. monocytogenes* but only at very high concentrations (10^8 per well) of heat-killed cells (data not shown). Slight inhibition has also been seen with very high concentrations of live non-LLO-producing *L. monocytogenes* (Cluff et al., submitted). This effect may be due to listerial proteins or carbohydrates other than LLO. Considering these observations, expression of recombinant LLO in *E. coli* has proved to be a good way to study the antigenicity and inhibitory activity of LLO without the complication of other interfering listerial moieties. Thus, the data in Fig. 8 suggest that recombinant LLO can inhibit presentation of antigen by macrophages. The mechanism of inhibition, however, remains unknown.

The multiple functions of LLO. The diverse activities of LLO present an interesting paradox. How can a membrane-active cytolysin act as a strong T-cell antigen as well as a virulence factor and potent inhibitor of antigen presentation?

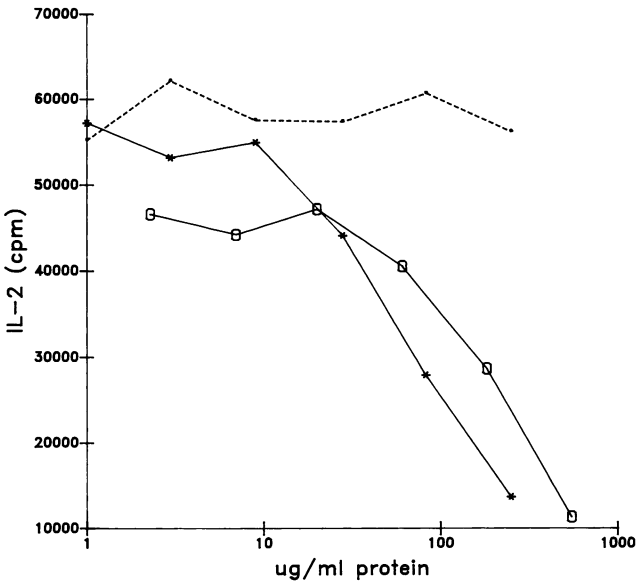


FIG. 8. Inhibition of antigen presentation by recombinant LLO. LLO in bacterial cell lysate of *E. coli* BB4(pT10e) (×), LLO in 50% ammonium sulfate precipitate fraction of culture supernatants from *L. monocytogenes* ATCC 43251 (○), and bacterial cell lysate of *E. coli* BB4(pBluescript) (---) were incubated with antigen-presenting cells and the antigen, OVA (100 μg per well), in microdilution plates. Macrophages were fixed, and responder T cells (OVA-specific DO11.10 hybridoma) were added to each well as described in Materials and Methods. Supernatants were collected 18 h later and evaluated for IL-2.

This issue is addressed in Table 5 by comparing the dose requirements for the activities of recombinant LLO present in lysates of *E. coli* BB4(pT10e). Our work indicates that *L. monocytogenes*-immune T cells became activated by a minimum dose of 0.3 μg/ml, whereas in vitro hemolytic activity was first detected at 4 μg/ml. Furthermore, the minimal effective dose of recombinant LLO preparations necessary to observe inhibition of antigen presentation in vitro was 20 μg/ml. As such, each of these activities is manifested at a different concentration. Because these quantitative relationships may be influenced in as yet undefined ways by the dynamics of the infectious process and other components present in these assay systems, these data should be interpreted with caution. Nonetheless, it is clear that antigenic activity is observed at concentrations about 100-fold less than those that are inhibitory for antigen presentation.

The paradox of LLO as a virulence factor and inhibitor of antigen presentation in addition to a major antigen can be most simply explained as a threshold effect. Under conditions in which large numbers of bacteria are present, the predominant effect may be inhibition of macrophage function. Such effective doses of bacteria may be reached either by a large infective dose or by uncontrolled replication of bacteria that is favored by the production of LLO (30). Inhibition of antigen presentation could effectively abrogate the generation of specific T cells that could aid in the clearance of intracellular bacteria (8), and the resulting uncontrolled replication of bacteria would result in death of the host. On the other hand, smaller numbers of LLO^+ bacteria may be effectively handled by innate defense reactions keeping the quantity of LLO below a critical threshold and permit antigen presentation and effective T-cell responses. Such responses would lead to the resolution of the infection and establishment of a state of immunity maintained by memory T lymphocytes.

TABLE 5. Activities of recombinant LLO[a]

Activity	Minimal effective dose	50% Effective dose
Antigen	0.3	2.0
Hemolysin	4.0	10.0
Inhibitor	20.0	60.0

[a] Values represent protein concentrations in micrograms per milliliter of preparations of recombinant LLO derived from *E. coli* BB4(pT10e) determined as shown in Fig. 6 and 8. Lysates of control bacteria without the LLO gene had none of these activities.

In summary, we identified recombinant LLO as a membrane-active cytolysin, an inhibitor of antigen presentation by macrophages, and an antigen important in the T-cell-mediated immune response to *L. monocytogenes*. We also identified non-LLO antigens as T cell epitopes. This analysis of listerial proteins and cloning of LLO and non-LLO antigens will likely facilitate further studies regarding the immunogenicity of these molecules and will be valuable in the study of immunity to this intracellular pathogen.

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