Listeriolysin O Is a Target of the Immune Response to *Listeria monocytogenes*

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Summary

The immunologic mechanism of protective immunity to the intracellular parasite *Listeria monocytogenes* (Lm) is not well understood, however, antilisterial immunity can be adoptively transferred with T lymphocytes from Lm-immune donors. The Lm-immune cells are believed to produce macrophage-activating lymphokines, which leads to the eventual macrophage-dependent eradication of the bacterium. Increasing evidence suggests that immunity to Lm resides exclusively within the $CD8^+$ T cell subset. It is possible that the Lm-immune $CD8^+$ T cells function to release sequestered Lm from nonprofessional phagocytes to awaiting activated macrophage populations. This study was conducted to determine if listeriolysin O (LLO), which is an essential determinant of Lm pathogenicity, is also a target of the antilisterial immune response. We have found that target cells infected with a LLO^+ Lm strain are lysed by Lm-immune cytotoxic cells, whereas target cells infected with a LLO^- Lm mutant, or pulsed with a heat-killed Lm preparation, are not lysed by the Lm-immune effector cells. We have used a *Bacillus subtilis* (Bs) construct that expresses the LLO gene product and found that target cells infected with the LLO^+ Bs construct are lysed by antilisterial cytotoxic cells. The antilisterial cytotoxic response is targeted against LLO, in that we have also used a Bs construct that expresses the perfringolysin (PLO) gene product and found that target cells infected with the PLO^+ Bs are not lysed by antilisterial cytotoxic effector cells. These data strongly suggest that LLO is a target antigen of antilisterial immunity and may represent the dominant target during the expression of the immune response to Lm.

Listeria monocytogenes (Lm)[1] is a facultative intracellular parasite that can grow intracytoplasmically within parenchymal cells as well as macrophages after escape from the phagolysosome (1–3). This escape has been directly associated with the production of listeriolysin O (LLO), a hemolysin produced by all virulent strains of Lm (4). Our previous finding that a recombinant strain of *Bacillus subtilis* (Bs), which expresses the LLO gene of Lm, acquired the ability to escape from the phagolysosome and replicate intracytoplasmically after phagocytosis by a macrophage-like cell line supports the role of LLO in Lm virulence (5).

Immunity to Lm is T cell dependent with the ultimate destruction of the parasite contingent on the appearance of activated macrophages (6, 7). Although $CD4^+$, $CD8^-$ T cells contribute to the development of antilisterial immunity, it has been demonstrated that the $CD4^-$, $CD8^+$ subset is the required effector T cell component for expression of high levels of adoptively transferred antilisterial immunity (8, 9). Cells of this latter subset often express antigen-specific class I–restricted cytotoxicity, and this restriction has been reported for expression of antilisterial immunity (10). It also has been reported that cytotoxicity and immunity to *Listeria* can be expressed in a non-MHC-restricted fashion (11). In either case, the identity of the *Listeria*-derived antigens that are targets of the protective antilisterial response remains unknown. In this report we demonstrate that antilisterial cytotoxic cells only recognize target cells that have been infected with bacteria of the LLO^+ phenotype.

[1] *Abbreviations used in this paper:* BFA, brefeldin A; BHI, brain heart infusion; Bs, *Bacillus subtilis*; HKLM, heat-killed *Listeria monocytogenes*; IPTG, isopropyl β-D-thiogalactopyranoside; Lm, *Listeria monocytogenes*; LLO, listeriolysin O; PLO, perfringolysin O.

Materials and Methods

Bacteria. Lm 10403 serotype 1 was originally obtained from the American Type Culture Collection (Rockville, MD). Virulence

is maintained by repeated passage in mice. The LD_{50} dose for strain 10403 is ~4.5 × 10^3 bacteria injected intravenously into BALB/c mice. Lm DP-L215 is a transposon-induced LLO^- mutant (12). Bs strain 980 (Bs980) has been transformed with the LLO gene, and only produces LLO in the presence of isopropyl β-D-thiogalactopyranoside (IPTG; Sigma Chemical Co., St. Louis, MO) Bs strain 979 (Bs979) is the transformant control for Bs980. These strains have been described previously (5). Bs strain 1512 (Bs1512) has been transformed with the perfringolysin O (PLO) gene from *Clostridium perfringens*, and Bs1512 only produces PLO in the presence of IPTG. Heat-killed Lm (HKLM) was prepared by incubating a brain heart infusion (BHI; Difco Laboratories, Detroit, MI) broth culture of log-phase Lm 10403 at 60°C for 60 min.

IPTG Induction of Hemolysin Expression by Bs980 and Bs1512. Streak plates of Bs980 and Bs1512 were prepared 24 h before IPTG exposure. A BHI broth suspension of the organism was prepared and diluted to an OD_{500} of 0.05 in 10 ml of BHI + 5 mM IPTG, and placed in a shaking 37°C water bath. After 2 h (mid-log phase growth), the organisms were used in the cytotoxicity assay. Supernatant material was collected from the IPTG-induced cultures for verification of LLO or PLO production. Hemolytic activity of the supernatants was determined by SRBC lysis.

Mice. 6-wk-old female BALB/c mice were purchased from Bantin-Kingman (Fremont, CA).

Immunization. 8-wk-old BALB/c mice were immunized with ~0.1 LD_{50} of viable Lm injected in a 0.2-ml volume via the tail vein.

Cell Culture. Spleen cells from mice immunized 6 d previously with Lm were stimulated in culture with 1.0 μg/ml Con A (Sigma Chemical Co.) in RPMI 1640 (Gibco Laboratories, Grand Island, NY) supplemented with 5% FCS (Tissue Culture Biologicals, Tulare, CA). A total of 10^6 lymphoid cells were cultured in 75-cm^2 flasks (Falcon Labware, Oxnard, CA) in 50-ml volumes. These spleen cells were cultured for 72 h and maintained at 37°C in humidified 7.5% CO_2 and 92.5% air.

Antibody Plus Complement Mediated Depletion of Splenic Lymphocyte Populations. Con A–stimulated immune spleen cells were suspended at 10^7 cells/ml in RPMI containing anti-Thy-1.2 (hybridoma 30-H12), anti-CD4 (hybridoma GK1.5), or anti-CD8 (hybridoma 19-178) at 4°C for 1 h with occasional mixing. The antibody-treated cell suspensions were pelleted and resuspended in Low Tox-M rabbit complement (Accurate Chemical & Scientific Corp., Westbury, NY) diluted 1:12 in RPMI containing 0.3% BSA. The cells were incubated at 37°C for 1 h with occasional mixing. The cell suspensions were pelleted, and viability was determined by nigrosine dye exclusion. The cell populations were analyzed by flow cytometry to confirm efficiency of specific subset depletion.

Determination of log_{10} Protection. Recipient mice were infused intravenously with 2–5 × 10^7 Lm-immune cells in a volume of 0.2 ml. 1 d after adoptive transfer, groups of mice (four to five per group) were challenged with a 10 L_{50} dose of viable Lm 10403 administered intravenously. The recipient spleens were removed 48 h after the challenge and individually homogenized in 4.5 ml of 1% proteose-peptone in PBS. The homogenates were serially diluted, and plated onto BHI agar. Log_{10} protection was determined by subtracting the mean log_{10} CFU/spleen of the test group from the mean log_{10} CFU/spleen of the normal control group.

Cytotoxicity Assay. J774 macrophage like target cells (American Type Culture Collection) were plated at 2 × 10^5 cells/well (24-well plate; Costar, Cambridge, MA) in antibiotic-free DMEM (Gibco Laboratories) supplemented with nonessential amino acids (Gibco Laboratories) and 5% FCS. 18 h later, the J774 monolayers were infected with either Lm 10403, Lm DP-L215, Bs980, Bs979, Bs2512, or pulsed with HKLM. After 60 min, the monolayers were washed three times with prewarmed sterile PBS, and after the last PBS wash, extracellular bacterial replication was prevented by the addition of 0.5 ml DMEM containing 20 μg gentamycin. If the J774 target cells were to be used in a chromium release assay, the J774 target cells were labeled with 30 μCi/well ^{51}Cr for 2 h. The J774 target cells were washed three times with PBS to remove excess ^{51}Cr, and 0.5 ml DMEM + 5% FCS + gentamycin was added after the final wash. Effector cell populations were added in a 0.5-ml volume 4 h after initiation of the infection to the ^{51}Cr-labeled or nonlabeled J774 target cell monolayers. The assay was terminated 4 h after the addition of the effector cell populations. For determination of specific ^{51}Cr release, the 24-well plates were centrifuged, and 500 μl of supernatant was removed for analysis. The percentage of specific ^{51}Cr release was calculated as (experimental release − background release)/(maximum release − background release). Intracellular bacteria were recovered by lysing the J774 target cells with 1 ml sterile distilled water. 5 min later, dilutions of the bacterial suspensions were plated onto BHI agar plates. The plates were incubated overnight at 37°C, and the number of CFU was determined. The percent reduction in Lm was determined by: viable Lm per well of target cells incubated with effector cells/viable Lm per well of target cells incubated in the absence of effector cells.

Brefeldin A (BFA). BFA was dissolved in methanol, and added to the Lm-infected J774 target cells at 0.5 μg/well 2 h after the initiation of the infection. The presence of BFA was maintained throughout the remainder of the assay period. In several experiments, BFA was removed by washing the infected J774 target monolayers three times with PBS before the addition of effector cell populations. BFA does not inhibit extracellular or intracellular replication of Lm.

Results and Discussion

In this investigation we used the J774 macrophage–like cell line infected with either Lm or hemolysin-producing constructs of Bs in an attempt to elucidate the role of LLO in the immune response to Lm. As reported previously (5), in the presence of IPTG, strain Bs980 is able to produce LLO. Consequently, after phagocytosis by the J774 cell line, IPTG-induced Bs980 is able to escape the phagolysosome and replicate intracellularly. In this study we also used a Bs construct, strain Bs1512, which we observed to produce PLO in the presence of IPTG, and which was also able to replicate within the J774 macrophage–like cell line. These two recombinant strains provided an opportunity to determine if LLO is a target of antilisterial cytotoxic cells. We have determined that the population of *Listeria*-immune spleen cells, which are obtained after stimulation in culture with the polyclonal T cell mitogen Con A, and which adoptively transfer enhanced levels of antilisterial immunity, also show an enhanced ability to lyse Lm-infected target cell populations. The J774 macrophage–like cell line has been used to describe various biological features of a Lm infection, and is used as the target cell population in this study (13).

As presented in Table 1, antilisterial cytotoxic cells (the cell population obtained after stimulation of *Listeria*-immune spleen cells in culture with Con A) lyse J774 targets infected with viable LLO-producing Lm. Antilisterial cytotoxic

Table 1. *Listeria-immune Spleen Cells Are Cytotoxic against Target Cells Infected with LLO-producing Listeria monocytogenes*

J774 target cells infected with*	Effector cell population‡	Addition of BFA§	Percent decrease of *Listeria*‖	Percent ^{51}Cr release¶
Lm 10403, LLO^+	Normal	–	26	22
	Lm immune	–	90	80
	Lm immune	+	2	NT
	Lm immune	+ –> – **	84	NT
Lm DP-L215, LLO^-	Normal	–	22	4
	Lm immune	–	17	8
HKLM	Normal	–	NT	2
	Lm immune	–	NT	12
Lm 10403, LLO^+	Lm immune ($CD8^+$)‡‡	–	94	NT
	Lm immune ($CD4^+$)	–	18	NT

* Cultures of the J774 target cells were infected with Lm from an overnight broth suspension. For the LLO^+ wild-type strain, the multiplicity of infection (MOI) was 0.2–0.5. For the LLO^- DP-L215 strain, the MOI was 10–20. The HKLM was added at ~50 organisms/J774 cell.
‡ Con A–stimulated normal spleen cells (normal) and Con A–stimulated immune spleen cells (Lm immune) were added to the J774 monolayers 4 h after initiation of Lm infection. These effector cell populations were added at an E/T cell ratio of 5:1 in a 0.5-ml volume.
§ BFA was added at 0.5 μg/well to the J774 monolayers 2 h after the initiation of the Lm infection.
‖ The assay was terminated 4 h after the addition of effector cell populations. At the final time point, the number of *Listeria*/well was determined as described in Materials and Methods. The data are presented as percent reduction in viable *Listeria*/well of target cells incubated with effector cells compared with *Listeria*/well of target cells incubated without effector cells. Data are representative of at least four independent experiments. NT, not tested.
¶ Target cells were cultured and infected as described above and in Materials and Methods. The percentage of specific ^{51}Cr release was calculated as (experimental release – background release)/(maximum release – background release).
** BFA was removed by washing at the time the effector cell populations were added.
‡‡ Antibody plus complement-mediated depletion was performed as described in Materials and Methods. Effector cells were added at an E/T cell ratio of 5:1.

cell–mediated lysis was not seen after J774 target cell phagocytosis of either the LLO^- DP-L215 strain of Lm or HKLM. Data in Table 1 also demonstrate that antilisterial cytotoxicity is a property of the $CD4^-$, $CD8^+$ T cell subset. Target cell development is dependent on an intracellular processing event that is inhibited in the presence of BFA, and this BFA-mediated inhibition of target cell lysis is reversible. BFA has been shown to inhibit endogenous pathway-dependent transport of antigen and accessory cell molecule to the cell surface (14). Other studies also have shown that *Listeria*-immune $CD4^-$, $CD8^+$ T cell–dependent recognition of target cells requires intracellular growth of LM, which is only seen with strains producing LLO (15). However, there are at least two reports indicating that $CD4^-$, $CD8^+$ T cell–mediated cytotoxicity occurs after phagocytosis of HKLM (11, 16), a finding that could not be duplicated in this study.

The results in Table 1 indicate that antilisterial cytotoxicity is observed only in target cells in which LLO production allows the intracellular replication of Lm, however, it is not clear if LLO is a target of the cytotoxic response. This possibility was addressed in a series of experiments in which the J774 cell line was infected with the various Bs constructs producing LLO (Bs980) or the heterologous hemolysin, PLO (Bs1512). The data in Table 2 show that J774 cells infected with IPTG-induced strain Bs980 are recognized and lysed by antilisterial cytotoxic cells. J774 cells infected with Bs980 in the absence of IPTG (and thus in the absence of LLO) are not targets for lysis by antilisterial cytotoxic cells. In addition, J774 cells infected with IPTG-induced Bs1512 (and thus PLO^+) are not lysed by antilisterial cytotoxic cells, even though the Bs1512 strain produces PLO and replicates freely within the cytoplasm of the J774 cell. Therefore, the stimulus provided solely by the presence of Bs within the cytoplasm of the host cell does not render such cells susceptible to lysis by the antilisterial cytotoxic cell populations used in these assays.

Previous reports have shown that LLO is a critical virulence factor and that cells of the $CD4^+$, $CD8^-$ T cell subset are produced during an immune response to Lm that recognize epitopes of LLO (17, 18). However, immunization with isolated LLO or immunogenic peptides does not produce immunity to subsequent infection with Lm, although a $CD4^+$, $CD8^-$ T cell response can be readily measured (18). The lack of immunity seen in LLO-immunized animals may be related to the requirement of $CD4^-$, $CD8^+$ T cells for full expression of antilisterial immunity (9, 19). Thus, if LLO is an important target of the antilisterial immune response, then immunization protocols ensuring a $CD4^-$, $CD8^+$ T cell response to LLO epitopes are required to identify the importance of this component in a primary antilisterial response.

Table 2. *Listeria-immune Cytotoxic Cells Recognize Listeriolysin*

Target cells infected with*	Hemolysin production‡	Effector cell population§	Percent decrease of bacteria‖
Lm 10403	LLO	Normal	18
		Lm immune	91
Bs979	-	Normal	28
		Lm immune	34
Bs979 + IPTG	-	Normal	17
		Lm immune	25
Bs980	-	Normal	19
		Lm immune	16
Bs980 + IPTG	LLO	Normal	22
		Lm immune	87
Bs1512 + IPTG	PLO	Lm immune	5

* The J774 cells were infected with the various strains of bacteria as described in Materials and Methods.

‡ Of the various strains of bacteria used to infect the J774 target cells, some constitutively produce LLO, while others can be induced to produce LLO or PLO after IPTG exposure.

§ Con A–stimulated normal spleen cells (normal) and Con A–stimulated immune spleen cells (Lm immune) were added to the J774 monolayers 4 h after initiation of infection. These effector cell populations were added at an E/T cell ratio of 5:1 in a 0.5-ml volume.

‖ The assay was terminated 4 h after the addition of effector cell populations. At the final time point, the number of bacteria/well was determined as described in Materials and Methods. The results are represented as percent reduction in viable bacteria/well of target cells incubated with effector cells compared with bacteria/well of target cells incubated without effector cells. Data are representative of at least four independent experiments.

In a recent report, it was shown that H-2^d *Listeria* immune cells lysed H-2^d target cells pulsed with a synthetic peptide that corresponds to the 91–99 region of the LLO protein (20). This nanomer fulfills the binding motif requirements for interaction with the H-$2K^d$ molecule. Although additional synthetic LLO molecule nanomers that fulfill H-$2K^d$ binding motif constraints were tested, only the 91–99 region was found to sensitize target cells for lysis. The in vivo activity of the antilisterial cytotoxic cell line population used for this study was not reported. Therefore, the importance of the 91–99 region of LLO as a target by cells capable of adoptively transferring antilisterial immunity is not known. Considering the intracellular biology of Lm, it is likely that LLO is a critical target of a protective antilisterial response. LLO is apparently required for the initial escape of the organism from the phagocytic vesicle (13, 21). Escape to the cytoplasm is an early event in the parasitic life cycle of Lm. Thus, the production of LLO within the phagolysosome may lead to peptide association with MHC molecules, transport to the target cell surface, and recognition by Lm-immune T cells. This would be analogous to the early proteins produced in virally infected cells that are also the targets of protective immune responses (22, 23). It is possible to consider that although many listerial antigens may be produced during the intracellular growth of this microbe, only a limited number of antigens are crucial for immunologic recognition by *Listeria*-immune cytotoxic cells. As a corollary, it is possible that recognition of a *Listeria*-infected cell by $CD4^-$, $CD8^+$ *Listeria*-specific T cells is limited to epitopes of the listeriolysin molecule. The data provided here demonstrate that LLO is one, if not the major, antigen associated with the immune T cell response in antilisterial immunity.

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