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The Role of the Bacterial Membrane Protein ActA in Immunity and Protection Against *Listeria monocytogenes*1

Ayub Darji,2* Dunja Bruder,* Susanne zur Lage,* Birgit Gerstel,* Trinad Chakraborty,† Jürgen Wehland,* and Siegfried Weiss*

ActA, an essential virulence factor of *Listeria monocytogenes*, is an integral membrane protein that is required for intracellular motility, cell-to-cell spread, and rapid dissemination of the bacteria in the infected host. To reveal cytotoxic T cell responses against ActA we introduced a recombinant soluble form of ActA into the MHC class I-processing compartment of APC using a variant of listeriolysin mutated within its immunodominant MHC class I epitope. With this experimental system we demonstrate that T cells are induced against ActA during a sublethal infection with *L. monocytogenes*. However, adoptively transferred cytotoxic CD8⁺ T cells specific for ActA did not protect mice against a subsequent challenge with this pathogen. This was due to an inability of APC to present ActA by either MHC class I or class II molecules as long as ActA remained tethered to the surface of intracellular viable bacteria. ActA was only presented when *L. monocytogenes* were engineered to secrete ActA or when the bacteria were killed by antibiotics during the assay. These findings raise questions on the general use of membrane proteins of pathogens as candidates for subunit vaccines. *The Journal of Immunology*, 1998, 161: 2414–2420.

T cells recognize Ag only when it is processed to small peptides and presented by MHC molecules on the surface of APC (1). The major pathways that exist for presentation by either MHC class I or class II molecules are fairly well understood. Since T cells are pivotal in the defense against pathogens, the Ags required for the generation of protective immune responses have to be made available to the processing and presentation machinery of the APC. This is clearly the case for most viral proteins, since these pathogens are largely dependent on the biochemical processes of the host cell. Thus, viral proteins are subject to the cellular degradation mechanisms and the Ag presentation that lead to virus-specific immune responses. As a consequence, viruses employ many different strategies to inhibit Ag processing and MHC class I-dependent presentation (2).

Unlike viruses, protein turnover in intracellular bacteria and parasites is conducted within the context of the pathogen, and it is questioned whether such proteins are available to the processing machinery of the host cell as long as the organism is viable. On the other hand, surface-bound and secreted proteins of the pathogen should be accessible to processing and could therefore result in T cell responses.

The facultative intracellular bacterium *Listeria monocytogenes* is the causative agent of listeriosis. It represents a well-studied pathogen in the mouse (3–5). Protective immunity to *L. monocytogenes* is exclusively exhibited by T cells, mainly of the CD8⁺ type (3–5), although recently CD4⁺ T cells also have been shown to be protective (6). Several secreted and surface-bound virulence factors of this pathogen that are involved in its uptake by non-phagocytic cells, escape from the phagolysosome, intracellular mobility, and cell-to-cell spreading have been identified and characterized (7, 8). Some of these virulence factors might represent protective Ags, but this has definitely been shown only for listeriolysin O (LLO), a secretory protein that is involved in escape of the bacterium from the vacuole (6, 9).

The listerial membrane protein ActA constitutes one of the major virulence factors of the pathogen (7, 8). It is found in high concentrations on the surface of virulent *Listeria* and is required for the recruitment of host cytoskeletal components to generate intracellular motility. Genetic and biochemical studies have led to the delineation of several functional regions within this molecule required for these interactions (8). Despite its importance in the infection cycle, it is not clear whether an immune response is mounted against this membrane protein during infection. In addition, it is unclear whether immunity against this protein would indeed protect mice against a subsequent challenge with *L. monocytogenes*.

The availability of transfectant cells expressing LLO has greatly facilitated the isolation and characterization of T cell subsets specific for that virulence factor (10). However, it has not been possible to obtain stable transfectants with the ActA structural gene, probably because expression of high levels of ActA is deleterious for normal host cell cytoskeletal function (11, 12). We, therefore developed a novel strategy to assess the induction and strength of the cytotoxic responses and to obtain cytotoxic CD8⁺ T cell lines against ActA. Capitalizing on our previous observation that the pore-forming activity of LLO can be used to introduce soluble proteins into the MHC class I-processing compartment and to stimulate CD8⁺ T cells in vivo and in vitro (13, 14), we purified a recombinant truncated form of ActA lacking its membrane and cell wall binding regions (B. Gerstel et al., unpublished observation) and used it as an Ag in LLO-mediated cytotoxic assays. In

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3 Abbreviations used in this paper: LLO, listeriolysin O; NP, nucleoprotein; LD₅₀, dose resulting in the death of 50% of the animals.
addition, cytotoxic T cells were raised against soluble ActA using the same strategy. To avoid a concomitant CD8⁺ T cell response against wild-type LLO, a fully hemolytically active variant of the toxin was used in which the single immunodominant epitope (10, 15) had been mutated.

Using these protocols we demonstrate that the listerial membrane protein ActA is immunogenic in mice that survive an infection of *L. monocytogenes*. However, cytotoxic CD8⁺ T cells against this virulence factor do not protect mice against a lethal challenge with the pathogen, whereas CD8⁻ T cells against the secretory LLO do. We present evidence indicating that as long as ActA is present on the membrane of the bacteria and as long as the intracellular bacteria remain viable, ActA is not available to the MHC class I and class II presentation mechanisms of the infected host APC.

**Materials and Methods**

**Mice**

Six- to eight-week-old female BALB/c mice obtained from Harlan Winkelmann (Borchen, Germany) were used in all studies.

**Bacterial strains**

XL-1-blue of *Escherichia coli* (Stratagene, San Diego, CA) was used as host for cloning and plasmid propagation. Bacteria were grown in Luria Bertoni medium or on Luria-Bertoni agar plates supplemented with 400 μg/ml erythromycin or 100 μg/ml spectinomycin. Where indicated, *L. monocytogenes* EGD was used for in vivo challenge and in vitro presentation assays and as the source of template DNA to amplify the derivatives of the *actA* gene. Either *L. monocytogenes* Δ*actA2*, harboring an in-frame deletion in the gene *actA* from codons for amino acids 20 to 602 (16), or *L. innocua* (American Type Culture Collection 33090, Rockville, MD), a nonpathogenic *Listeria* species, was used as the recipient for plasmids that express truncated ActA. *L. monocytogenes* Δ*actA2* contains an in-frame deletion in the structural gene of LLO from codons of amino acids 61 to 420 (16). Listeria spp. were grown using brain-heart infusion (Difco, Detroit, MI) broth or agar supplemented with either 5 μg/ml erythromycin or 100 μg/ml spectinomycin where required.

**Recombinant DNA techniques**

DNA preparation, genetic manipulations, and PCR were conducted according to standard protocols (17). Transformation of bacteria was performed as described by Hanahan (18) or by electroporation (19). DNA sequencing was conducted with the Taq Dye Deoxynucleotide terminator cycle sequencing system (Applied Biosystems, Foster City, CA) and analyzed on an Applied Biosystems 373A automated DNA sequencer.

**Listeria secreting soluble ActA**

*L. monocytogenes* actAΔ in which the *actA* structural gene has been interrupted by the insertion of a gene imparting erythromycin resistance results in the expression of a truncated ActA protein comprising amino acids 1 to 425. The leader peptide from amino acids 1 to 30 is removed upon membrane transport. Thus, *L. monocytogenes* actAΔ secretes soluble ActA of amino acids 31 to 425 (20). *L. monocytogenes* Δ*actA2* harboring plasmid pactA₁₋₅₀ᵉ, hereafter designated *L. monocytogenes* actA₅₀⁶, was constructed using chromosomal DNA as template and comprised a truncated version of *actA* containing the codons for amino acids 1 to 506 in addition to its own promotor. The fragment was amplified with the primer pair 5'GAACGTGGACATCCTTACGGG-3' and 5'-AGCTTTGGATCCTCTTTTACGGG-3' containing a XhoI and a BamHI restriction site, respectively (underlined). Using these sites, the amplification product wasdigested vector pERL3/50-1 (22). This vector contains the positive regulatory factor PrfA, allowing hyperexpression of genes under the control of PrfA-dependent promoters. Thus, *L. innocua* actA represents a noninvasive transformant secreting ActA of amino acids 31 to 506.

**Cell lines**

The macrophage-like cell line J774, which expresses MHC class I and class II, and the class II-negative mastocytoma P815 were used as stimulator or target cells in T cell assays. The CD8⁺ T cell clone HASL, specific for the nucleoprotein (NP) of influenza virus, was a gift from Dr. Rammensee (Tübingen, Germany).

**Generation of T cell lines and hybridomas**

A cytotoxic CD8⁺ T cell line against LLO was induced by immunizing mice with soluble active LLO in IFA, which induces CD8⁺ T cells in addition to CD4⁺ T cells (13). This is due to the ability of LLO to form pores in the cellular membrane, thus generating access to the MHC class I presentation pathway. Nine days after immunization, spleen cells were harvested and restimulated with the immunodominant MHC class I peptide comprising amino acid positions 91 to 99 of LLO (10, 15). After three cycles of in vitro restimulation, T cells were analyzed for cell surface markers using cytofluorometry and for Ag specificity. The pore-forming activity of LLO was also used for induction of a CD8⁺ cytotoxic killer line specific for ActA. To avoid a concomitant stimulation of CD8⁺ T cells against LLO we used a variant in which Thy1 was replaced by Ala. This variant is fully active hemolytically but does not stimulate CD8⁺ T cells in H2d mice (D. Bruder et al., unpublished observations). A mixture of soluble ActA with LLO/92A was injected i.p. in IFA, and 9 days later spleen cells were stimulated with mitomycin C-treated P815 that had been pulsed with a mixture of ActA and LLO/92A for three cycles of 10–12 h intervals of 10 to 12 days, T cells were analyzed for cell surface markers and specificity, and then used in the in vivo protection or in vitro presentation assays.

To measure presentation of ActA by MHC class II, a T cell hybridoma specific for ActA was established (A. Darji et al., unpublished observations). Mice were immunized with soluble ActA in IFA, and spleen cells were fused with BW 5147 after one restimulation with soluble ActA in vitro.

**CTL assays**

The JAM assay was used for all cytotoxic tests (23). P815 and J774 cells were labeled with ³⁵S]methionine for 3 to 4 h or for 14 h, respectively. To reveal cytotoxic activity against LLO, labeled P815 cells were pulsed with 10 μM LLO peptide 91–99 and incubated with spleen cells from immunized mice at an E:T ratio of 50:1. To reveal ActA-specific cytotoxic T cells, labeled P815 cells were pulsed with a mixture of soluble ActA and LLO/92A before incubation with spleen cells from immunized mice at an E:T cell ratio of 50:1. Assays were incubated for 4 h at 37°C, after which surviving cells were harvested on filter mats and estimated in a scintillation counter. Data were expressed as percent lysis.

To measure in vitro presentation of ActA, 1 × 10⁶ labeled J774 cells were distributed into wells of microtiter plates, allowed to adhere, and infected with 1 × 10⁶ wild-type or recombinant *L. monocytogenes* or *L. innocua*. After 30 min plates were gently washed, and fresh medium containing 10 μg/ml gentamycin was added to kill bacteria that were not phagocytosed. Finally, the ActA-specific cytotoxic T cell line was added with or without previous addition of 10 μg/ml ampicillin. Cytotoxicity was determined as described. Spontaneous lysis was <15% in all assays.

**MHC class II presentation assay**

After adherence in wells of microtiter plates, 2 × 10⁴ J774 cells were infected with 2 × 10⁵ *Listeria* of various strains for 30 min. Cultures were subsequently washed and supplemented with medium containing 10 μg/ml gentamycin, and then 5 × 10³ ActA-specific T cell hybridomas were added. As a control, T cells were incubated with untreated J774 in the presence of 1 μg/ml soluble ActA. After incubation for 24 h, 100 μl of culture supernatant was carefully removed, and T cell stimulation was determined by quantifying IL-2 production using proliferation of the indicator T cell line CTLL-2 as the read out.

**Flow cytometry**

Aliquots of the T cells used in the in vivo or in vitro assays were analyzed in parallel by flow cytometry. Cells were stained with Abs against TCR ββ (biotinylated; clone H57-59.7), CD4 (FITC; RM 4.5; PharMingen, San Diego, CA), CD8 (phycoerythrin; 53-6.7; PharMingen), and streptavidin (FITC or phycoerythrin; PharMingen).
In vivo protection assay

The ActA-, LLO-, and NP-specific cytotoxic T cells were harvested 10 to 12 days after the last restimulation. Mice were injected i.v. with 5 x 10^7 T cells in 200 μl of PBS. Thirty to sixty minutes later the mice were infected i.v. with 5 x 10^7 L. monocytogenes EGD (1 x LD₅₀). Organs were removed 72 h after infection and homogenized in sterile H₂O containing 0.2% Nonidet P-40. Serial 10-fold dilutions were plated, and colonies were counted after overnight incubation at 37°C. The detection limit was 20 CFU/organ. Student’s t test was employed for statistical analysis. Each experimental group consisted of four or five mice, and the experiment was performed a second time using 15 x 10^7 T cells with identical results.

To determine that the ActA-specific CD8⁺ T cells behave the same way in vivo as the LLO-specific CD8⁺ T cells, the variant strain L. monocytogenes actA1, which secretes a truncated form of ActA, was employed. Since this strain is attenuated for virulence, infection was conducted with 7.5 x 10^7 bacteria of the variant strain. Control mice were injected with 5 x 10^7 organisms of the wild-type strain. Protection was assessed 48 h after injection of the CD8⁺ T cells. The experiment was performed twice with identical results.

Results

The listerial membrane protein ActA is immunogenic in mice that survive a L. monocytogenes infection

To investigate the immune response against L. monocytogenes, BALB/c mice were infected with 1 x 10⁶ bacteria (0.2 x LD₅₀). After recovery, spleen cells of these mice were tested at 12 or 20 days postinfection. The immune response against LLO was first examined, since LLO is known to induce CD8⁺ as well as CD4⁺ T cells (10, 24–26). Cytotoxic anti-LLO activity could be revealed using target cells that were sensitized with the immunodominant peptide comprising amino acids 91 to 99 of LLO (Fig. 1A). This response was specific, since a H-2Kd-binding control peptide derived from the nucleoprotein of influenza virus was not recognized. Similarly, a peptide variant of LLO₉₁₋₉₉ in which the anchor residue Tyr⁹² was changed to Ala (pLLO₉₂A) was also not recognized. This finding suggested that hemolytic LLO variants with exchanges at the appropriate sites can be used in LLO-mediated MHC class I presentation assays of soluble proteins, since the variants themselves are not presentable by H-2Kd molecules.

The same spleen cells were used to test for cytotoxic activity against ActA. Figure 1B demonstrates that cytotoxic T cells against the membrane protein ActA are induced during an infection with L. monocytogenes. Target cells that were sensitized with a mixture of soluble ActA and a variant of the LLO protein in which Tyr⁹² had been mutated to Ala (pLLO₉₂A) were readily killed by splenic T cells from recovered mice. As expected from the data presented above, cells sensitized with LLO₉₂A alone or a mixture of LLO₉₂A and an irrelevant protein were not recognized by these T cells. Thus, by using an appropriate variant of LLO it was possible to reveal the cytotoxic response against ActA.

Spleen cells from recovered mice were also tested for proliferative responses against the soluble proteins ActA and LLO. These responses are mainly due to CD4⁺ T cells. As shown in Figure 1, C and D, both proteins elicit specific proliferation. Thus, after recovery from L. monocytogenes infection, CD4⁺ as well as CD8⁺ T cells against both the secreted protein LLO and the membrane-bound protein ActA were detected.

Specific cytotoxic T cells can be generated with a mixture of soluble ActA and variant listeriolysin

To examine whether cytotoxic T cells specific for ActA would protect mice from a lethal infection with L. monocytogenes, a cytotoxic T cell line against the truncated soluble form of the listerial membrane protein ActA was produced. Mice were immunized with a mixture of truncated ActA and LLO₉₂A. Since this variant of LLO possesses pore-forming activity identical with that of the wild-type LLO, it was possible to introduce ActA into the MHC class I presentation pathway, first in vivo and subsequently in vitro, without stimulating cytotoxic T cells against LLO at the same time. To avoid a CD4⁺ T cell co-response, splenic T cells of immunized mice were restimulated with pulsed P815 cells that do not express MHC class II molecules. Untreated spleen cells were added as feeders. Figure 2 shows that the CD8⁺ T cell line that resulted after three cycles of restimulation with this mixture was specific for ActA. As expected, the LLO variant LLO₉₂A was not recognized. LLO₉₂A, however, was required for introduction of ActA into the class I presentation compartment, since addition of ActA alone did not sensitize the target cells (Fig. 2).

Cytotoxic T cells against the membrane protein ActA do not protect mice against infection with L. monocytogenes

To assess the role of cytotoxic T cells against the membrane protein ActA in protection of the host against L. monocytogenes infection, the T cell line specific for ActA was tested in vivo. For comparison, an LLO-specific T cell line that had been established and characterized in parallel (data not shown) was used. As shown previously (9), cytotoxic T cells specific for the secreted toxin...
LLO dramatically reduced the bacterial load in spleen and liver (Fig. 3, A and B). Cytotoxic T cells against the membrane protein ActA did not influence the course of infection. No difference was observed between such mice and untreated mice or mice injected with a control cytotoxic T cell clone specific for the nucleoprotein of influenza virus.

The difference between the two T cell lines specific for either LLO or ActA might have been due to a difference in the composition of subpopulations or a difference in the in vivo behavior of the T cells. On the other hand, ActA associated with the bacterial membrane might not be presented to T cells by infected APC. We, therefore, repeated the above experiments and included the variant strain \( L.\ monocytogenes\ actA\) that secreted a truncated version of ActA. The T cells generated against ActA provided protection against \( L.\ monocytogenes\ actA\) to the same extent as LLO-specific T cells (Fig. 4B). In contrast, protection against the wild-type \( L.\ monocytogenes\ EGD\) was only provided by the LLO-specific T cells, as shown above (Fig. 4A). In parallel, T cells were examined by cytofluorometry. The ActA-specific T cell line was

**FIGURE 3.** Adoptive transfer of ActA-specific CD8\(^+\) T cells does not protect mice against a lethal infection with \( L.\ monocytogenes\). BALB/c mice were injected i.v. with either wild-type \( L.\ monocytogenes\ EGD\) or \( L.\ monocytogenes\ actA\) that secreted a truncated form of ActA. Spleens and livers were removed after 48 h and examined for CFU as described in Figure 3. Student’s \( t\) test was employed for statistical analysis, and the \( p\) values compared with untreated controls are indicated (*). No differences were observed when reduction of the bacterial burden of \( L.\ monocytogenes\ actA\) was compared from mice injected with either LLO- or ActA-specific CD8\(^+\) T cells (Fig. 4B; spleen, \( p > 0.18\); liver, \( p > 0.44\)). C, Analysis of ActA-specific T cells by flow cytometry.

**FIGURE 4.** Adoptive transfer of ActA-specific CD8\(^+\) T cells strongly reduces the bacterial burden in mice infected with an \( L.\ monocytogenes\) variant that secretes ActA. Mice were injected with either \( 5 \times 10^3\) CD8\(^+\) T cells specific for either LLO or ActA and infected 1 h later with either \( 5 \times 10^3\) \( L.\ monocytogenes\ EGD\) (A) or \( L.\ monocytogenes\ actA\) (B) that secreted a truncated form of ActA. Spleens and livers were isolated and homogenized, and bacteria were plated in serial dilutions to enumerate CFU. The \( p\) values for comparisons with untreated controls were determined by Student’s \( t\) test.

**FIGURE 2.** Specificity of CD8\(^+\) cytotoxic T cells against ActA. Mice were immunized i.p. with a mixture of 1 \( \mu g\) of variant LLO92A and 2 \( \mu g\) of soluble ActA in IFA. Nine days later immune spleen cells were isolated and restimulated with P815 cells pulsed with mixtures of LLO92A and soluble ActA for 10 to 12 days. After three cycles of restimulation, the specific cytotoxicity of T cells against ActA was analyzed using \([3\text{H}]\)thymidine-labeled P815 as target cells incubated with LLO92A mixed with ActA (ActA+LLO92A), LLO92A alone, ActA alone, or an irrelevant peptide (pNP). The E:T cell ratio was 10:1. All assays were performed in triplicate, and the SEM never exceeded 10%.

**FIGURE 3.** Adoptive transfer of ActA-specific CD8\(^+\) T cells does not protect mice against a lethal infection with \( L.\ monocytogenes\). BALB/c mice were injected i.p. with a mixture of 1 \( \mu g\) of variant LLO92A and 2 \( \mu g\) of soluble ActA in IFA. Nine days later immune spleen cells were isolated and restimulated with P815 cells pulsed with mixtures of LLO92A and soluble ActA for 10 to 12 days. After three cycles of restimulation, the specific cytotoxicity of T cells against ActA was analyzed using \([3\text{H}]\)thymidine-labeled P815 as target cells incubated with LLO92A mixed with ActA (ActA+LLO92A), LLO92A alone, ActA alone, or an irrelevant peptide (pNP). The E:T cell ratio was 10:1. All assays were performed in triplicate, and the SEM never exceeded 10%.

**FIGURE 2.** Specificity of CD8\(^+\) cytotoxic T cells against ActA. Mice were immunized i.p. with a mixture of 1 \( \mu g\) of variant LLO92A and 2 \( \mu g\) of soluble ActA in IFA. Nine days later immune spleen cells were isolated and restimulated with P815 cells pulsed with mixtures of LLO92A and soluble ActA for 10 to 12 days. After three cycles of restimulation, the specific cytotoxicity of T cells against ActA was analyzed using \([3\text{H}]\)thymidine-labeled P815 as target cells incubated with LLO92A mixed with ActA (ActA+LLO92A), LLO92A alone, ActA alone, or an irrelevant peptide (pNP). The E:T cell ratio was 10:1. All assays were performed in triplicate, and the SEM never exceeded 10%.
FIGURE 5. Lack of MHC class I presentation of membrane-associated ActA on viable *L. monocytogenes*. A, [3H]thymidine-labeled J774 target cells were infected in vitro at a multiplicity of infection of 10 with *L. monocytogenes* EGD (Lm), *L. monocytogenes* expressing secretory variants of ActA (Lm. actA1 and Lm. actA506), *L. innocua* (Linn.), or *L. innocua* expressing secretory ActA (Linn. actA506). Thirty minutes later cells were washed with gentamycin-containing medium and were cultured in gentamycin-containing medium for further 2 to 3 h before ActA-specific CD8+ T cells were added. In one culture (L. m. + Amp.) ampicillin was added to J774 cells that were infected with wild-type *L. monocytogenes* EGD 1 h before the cytotoxic assay to kill intracellular bacteria. As a positive control, soluble ActA was introduced into the MHC class I presentation pathway via purified LLO (LLO + ActA). Cytotoxicity was tested by JAM assay at E:T cell ratios of 10:1. B, J774 cells were infected with different strains of *L. monocytogenes* as described in A. As an additional control, *L. monocytogenes* ΔactA2 was included. This strain bears an in-frame deletion in the actA gene. Cytotoxicity was assessed at different E:T cell ratios in a 4-h JAM assay. C, Assay conditions were identical with those in B, except that ampicillin was added before addition of ActA-specific T cells. All assays were performed in triplicates and the SEM never exceeded 10%.

almost exclusively composed of CD8+ T cells that expressed an αβ TCR (Fig. 4C). Thus, the ActA-specific T cells consist of effector cells that have the same composition as the LLO-specific T cells. In addition, their in vivo activity is similar to that of the LLO-specific T cell line, provided that ActA is secreted by the bacteria.

The membrane protein ActA on viable bacteria is not presented by MHC class I molecules or class II molecules

The most likely explanation for the above finding is that ActA on viable *L. monocytogenes* is not accessible to the processing apparatus of the host cells; indeed, this turned out to be the case. Hence, J774 cells that were infected with live *L. monocytogenes* EGD were not killed by the ActA-specific cytotoxic T cell line in a 4-h cytotoxicity assay (Fig. 5A). These cells were, on the other hand, able to present ActA when it was introduced into the MHC class I pathway as a soluble protein with the help of LLO. More importantly, J774 cells infected with *L. monocytogenes* strains that produced soluble variants of ActA were also killed by the T cell line. Thus, ActA in principle can be processed and presented during *Listeria* infection. However, as long as it is associated with the bacterial membrane, it does not appear to be available for the processing mechanism. Interestingly, when bacteria were killed by addition of ampicillin at the beginning of the cytotoxic assay, ActA also became presentable despite being expressed on the bacterial membrane. Soluble ActA secreted by the noninvasive species *L. innocua* was not presented by J774. These bacteria do not escape from the phagolysosome, and as a result ActA does not become accessible to the MHC class I presentation pathway.

Since the T cell line specific for ActA was induced with a soluble form of ActA derived from supernatants of recombinant *L. monocytogenes* ΔactA2, we wanted to assure that the specificity of this line was exclusively directed toward ActA and not against potential contaminants. The in vivo protection experiment displayed in Figure 4B already strongly argues against such a possibility. To corroborate this finding, we infected J774 cells with the wild-type strain or the mutant *L. monocytogenes* ΔactA2 that bears an in-frame deletion in the ActA gene but is otherwise completely isogenic to the EGD wild-type strain. As Figure 5, B and C, demonstrates, J774 cells infected with the deletion mutant are not recognized by the ActA-specific killer cells regardless of whether ampicillin is added to the assay. On the other hand, J774 infected with the EGD strain is recognized as soon as the cells are treated with the antibiotic. This argues strongly for the monospecificity of the ActA-specific T cell line, since reactivity against listerial components other than ActA should have been revealed by these experiments. In addition, using a similar strategy and various isogenic mutant strains that contained small deletions in the actA gene, the epitope recognized by the ActA-specific T cell line was mapped. All reactivity was confined to a central region of the protein that showed an accumulation of peptides with Ld binding motifs (D. Bruder et al., unpublished observations). Thus, the ActA-specific CD8+ T cell line most likely recognizes one or more conventional peptides presented by Ld.

Similar results were obtained when presentation of ActA by MHC class II molecules was investigated. For this experiment, a specific CD4+ T cell hybridoma was established against soluble ActA (data not shown). Figure 6 demonstrates that soluble ActA can be presented very well to CD4+ T cells by this particular clone of J774. However, when ActA is expressed as a membrane protein on intracellular viable *L. monocytogenes*, it cannot be presented by MHC class II molecules. In contrast, J774 cells infected with *Listeria* that secrete ActA can present this Ag. When a mutant of *L. monocytogenes* was used that does not express LLO and thus is unable to escape from the phagolysosome, MHC class II presentation was observed. These bacteria are usually killed within these phagocytic compartments, and ActA on the bacterial surface thus becomes available to cellular processing mechanisms. This resembles the situation described above, whereby ActA became available for presentation by class I when the bacteria were killed by ampicillin treatment. As expected, ActA secreted by noninvasive *L. innocua* that remain in the vacuoles is presented very well (Fig. 6). Thus, the lack of protection can be explained by an absence of presentation of ActA early in the course of infection.
infection have been elucidated (8, 28). It is likely that all essential virulence factor of this pathogen. Several steps involved crofilament proteins that are required to facilitate intracellular soma, ActA is heavily phosphorylated (27) and is recruiting mi-

tify for motility of the bacterium within the host cell and for cell-to-cell stimulation of CD8

soluble ActA together with a variant of listeriolysin that no longer established in our laboratory (13, 14). Immunization with purified, protective immune response by using a strategy that had been recently

possible to establish stable transfectants of murine cells expressing of the actin-based cytoskeleton is probably the reason why it is not possible to establish stable transfectants of murine cells expressing ActA for use in immunologic assays (11, 12).

of protection in mice that were infected with L. monocytogenes (33). A 36-kDa intracellular protein isolated by expression cloning constituted a protective Ag, while the surface-exposed membrane protein gp63 did not. Taken together, these results cau-

Discussion

The membrane protein ActA of L. monocytogenes is responsible for motility of the bacterium within the host cell and for cell-to-cell spreading (20, 27). Upon escape of the pathogen from the phagolysosome, ActA is heavily phosphorylated (27) and is recruiting microfilament proteins that are required to facilitate intracellular movement of the bacterium (11, 12). Thus, ActA represents an essential virulence factor of this pathogen. Several steps involved in the subversion of the cytoskeleton of the host cell after L. monocytogenes infection have been elucidated (8, 28). It is likely that all or most of the ActA molecules expressed on the bacterial surface are engaged in recruiting and binding different components of the host cytoskeleton at the various stages of intracellular bacterial mobility. The apparent indiscriminate recruitment of components of the actin-based cytoskeleton is probably the reason why it is not possible to establish stable transfectants of murine cells expressing ActA for use in immunologic assays (11, 12).

Nevertheless, it was possible to test the role of ActA in a protective immune response by using a strategy that had been recently established in our laboratory (13, 14). Immunization with purified, soluble ActA together with a variant of listeriolysin that no longer stimulates CD8+ T cells in BALB/c mice allowed the establishment of an ActA-specific cytotoxic T cell line. Despite its strong cytotoxic activity, this T cell line was not protective in vivo. In contrast, a T cell line specific for the secretory LLO, raised under identical conditions, afforded protection to mice challenged with L. monocytogenes. It is possible that the T cell lines differed in their in vivo properties, i.e., in homing or survival. However, ActA-specific T cells provide protection that is equal in strength to that provided by LLO-specific T cells as soon as mice are infected with a variant strain of L. monocytogenes that secretes ActA. This clearly demonstrates their in vivo functional activity. In agreement with the findings reported here, we recently demonstrated the lack of protection in mice that were immune to ActA using a novel genetic immunization protocol with an attenuated Salmonella car-

ActA, mice were not protected against a lethal challenge with L. monocytogenes (29).

The lack of protection by ActA-specific cytotoxic T cells is most likely due to a lack of presentation of this membrane-bound bacterial protein. In vitro experiments clearly demonstrated that ActA, when expressed as a membrane protein, is not presented by either MHC class I or class II molecules, while a secreted form of ActA can be presented very well. This shows that the infection per se is not inhibiting the presentation of ActA. Interestingly, even when expressed on the surface of the pathogen, ActA can become available to the presentation mechanisms provided the bacterium is destroyed. This probably explains why in mice that have survived a Listeria infection, ActA-specific T cells can be demonstrated. Under these circumstances bacteria are destroyed by activated macrophages late in the course of infection. These cells might, in turn, be able to present ActA to both types of T cells.

One reason for the lack of presentation of ActA could relate to its phosphorylation by host cell kinases within the infected cell (27). However, we consider this unlikely because presentation is also not observed by MHC class II molecules. In this case, processing should take place in phagocytic vesicles, i.e., before ActA is phosphorylated. Although such an explanation for the inhibition of MHC class I presentation cannot formally be excluded, other explanations appear more likely. These include the stability of the bacterial protein within the host cell, which has been estimated to be >20 h (27). In addition, ActA, when tethered to the bacterium, is generally covered by recruited host cell cytoskeletal proteins that could protect the protein from degradation by the host cell. Thus, this protein would only be made available to processing mechanisms when it is present in a secreted form or after lysis of the bacteria.

Protection against L. monocytogenes is mainly provided by CD8+ T cells (3), but non-MHC class I-restricted T cells (25) as well as γδ T cells (30) have also been shown to contribute to protection. To date, only a few protective Ags that are recognized by CD8+ T cells have been defined (6, 9, 31, 32). LLO and p60 are both secretory proteins, whereas superoxide dismutase represents an intracellular enzyme. How intracellular proteins participate and contribute to a protective immune response whereas a membrane protein, such as ActA, cannot is unclear. However, similar results have been observed with the intracellular parasite Leishmania ma-

ior (33). A 36-kDa intracellular protein isolated by expression cloning constituted a protective Ag, while the surface-exposed membrane protein gp63 did not. Taken together, these results caution against the assumption that proteins exposed on the surface of the infecting microorganism are generally suitable candidates for the development of vaccines.

In principle, membrane proteins are attractive target Ags for vaccine development. The presence of serum Abs should result in a rapid clearance of many pathogens due to activation of the complement cascade or opsonization. However, in a situation where Th cells are required for the induction of Ab secretion by memory B cells or where T cells alone provide protection, as in listeriosis (34, 35), a subunit vaccine should induce immunity to an Ag that is available to T cells from the beginning of infection. Our results suggest that certain physiologic properties of the molecule involved, such as stability, post-translational modifications, or interaction with host cell components, could compromise the use of these proteins as Ags. Currently, these properties can be assessed by empirical studies only. The strategy described here should be of great help in determining the efficacy of these Ags as potential vaccine candidates.
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References