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Pathogenicity and Immunogenicity of a *Listeria monocytogenes* Strain That Requires D-Alanine for Growth

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Listeria monocytogenes **is an intracellular bacterial pathogen that elicits a strong cellular immune response following infection and therefore has potential use as a vaccine vector. However, while infections by *L. monocytogenes* are fairly rare and can readily be controlled by a number of antibiotics, the organism can nevertheless cause meningitis and death, particularly in immunocompromised or pregnant patients. We therefore have endeavored to isolate a highly attenuated strain of this organism for use as a vaccine vector. D-Alanine is required for the synthesis of the mucopeptide component of the cell walls of virtually all bacteria and is found almost exclusively in the microbial world. We have found in *L. monocytogenes* two genes that control the synthesis of this compound, an alanine racemase gene (*dal*) and a D-amino acid aminotransferase gene (*dat*). By inactivating both genes, we produced an organism that could be grown in the laboratory when supplemented with D-alanine but was unable to grow outside the laboratory, particularly in the cytoplasm of eukaryotic host cells, the natural habitat of this organism during infection. In mice, the double-mutant strain was completely attenuated. Nevertheless, it showed the ability, particularly under conditions of transient suppression of the mutant phenotype, to induce cytotoxic T-lymphocyte responses and to generate protective immunity against lethal challenge by wild-type *L. monocytogenes* equivalent to that induced by the wild-type organism.**

Listeria monocytogenes is a gram-positive facultative intracellular microorganism which has been used for decades as a model pathogen for the study of cell-mediated immunity (24). Immunization of mice with a sublethal *L. monocytogenes* infection results in the generation of immunity which is largely major histocompatibility complex (MHC) class I mediated. Such infections generate $CD8^+$ T cells, which can adoptively transfer immunity and specifically recognize and kill *Listeria*-infected target cells (3, 6, 17, 19).

Recently, the cell biology of *L. monocytogenes* intracellular growth has been defined (47). Subsequent to internalization, the bacteria escape from a phagocytic vacuole and replicate in the host cell cytosol. Hence, secreted proteins of *L. monocytogenes* are delivered directly into the cytosol and into the MHC class I pathway of antigen processing and presentation (1, 5). Mutants of *L. monocytogenes* which are unable to enter the cytosol are absolutely avirulent and fail to immunize mice, and cells infected by such mutants are not recognized by *L. monocytogenes*-immune $CD8^+$ T cells (6, 28).

The natural properties of *L. monocytogenes* make it particularly attractive as a potential live vaccine vector for the induction of cell-mediated immunity to foreign antigens. Indeed, recombinant *L. monocytogenes* expressing such antigens successfully has been used to protect mice against lymphocytic choriomeningitis virus (15, 40) and influenza virus (22) infections and against lethal tumor cell challenge (32, 33). We have suggested the use of *L. monocytogenes* for the induction of cytolytic T cells directed against human immunodeficiency virus (HIV) antigens and have shown that strong cell-mediated immune responses against HIV-1 Gag protein can be induced in mice infected with recombinant *L. monocytogenes* carrying a chromosomal copy of the HIV-1 *gag* gene (13).

Because of the potential broad use of this organism as a vaccine vector in infectious disease and cancer, the safety of *L. monocytogenes* becomes an important issue. While infections by *L. monocytogenes* are fairly rare and can readily be controlled by a number of antibiotics, the organism can nevertheless cause meningitis and death, particularly in immunocompromised or pregnant patients. An ideal vaccine strain of *L. monocytogenes* would be absolutely avirulent but fully immunogenic. We therefore sought to isolate a mutant which could enter the cytosol but have limited growth potential both in vivo and in the environment.

D-Alanine is required for the synthesis of the mucopeptide component of the cell walls of virtually all bacteria, including *L. monocytogenes* (21, 23, 43), and is also found in the lipoteichoic acids of this and some other gram-positive organisms (11, 37). However, it is present in only trace quantities and fails to accumulate in vertebrates; the likely origin of these trace quantities is the breakdown products of intestinal and food bacteria (16, 20, 25, 29). We hypothesized that a strain of *L. monocytogenes* that is unable to synthesize this compound could be grown in the laboratory when supplemented with D-alanine but should be unable to grow outside the laboratory, particularly in the cytoplasm of eukaryotic host cells, the natural habitat of this organism during infection. The isolation of such a mutant of *L. monocytogenes* required the identification and inactivation of two genes, *dal* and *dat*. *dal* encodes alanine racemase, which catalyzes the reaction: L-alanine↔D-alanine. *dat* en-

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codes D-amino acid aminotransferase, which catalyzes the reaction D-glutamic acid + pyruvate↔α-ketoglutaric acid + D-alanine. The *dal dat* double-mutant strain had the anticipated phenotype and in addition showed the ability to induce cytotoxic T-lymphocyte (CTL) responses and to generate protective immunity against lethal challenge by wild-type *L. monocytogenes* in infected mice under restricted conditions.

MATERIALS AND METHODS

Bacteria and plasmids. *L. monocytogenes* 10403S (34) was the wild-type organism used in most of these studies. It was grown in brain heart infusion medium (BHI; Difco Laboratories). *Escherichia coli* DH5α, used for cloning, was grown in Luria broth (38). Plasmid pKSV7, used for allellic exchange reactions in *L. monocytogenes*, is a shuttle vector capable of replication in *E. coli*, where it is selected in the presence of 50 μg of ampicillin per ml of medium, and in *L. monocytogenes*, where its replication is temperature sensitive and selection is in the presence of 10 μg of chloramphenicol per ml of medium (42). Plasmid DNA from *E. coli* and total DNA (chromosomal and plasmid) from *L. monocytogenes* were isolated by standard methods (38).

Identification of genes in *L. monocytogenes* by homology. Based on sequences of alanine racemase (*dal*) genes in two gram-positive organisms, *Bacillus subtilis* (10) and *B. stearothermophilis* (46), we devised consensus oligonucleotide sequences from highly conserved regions at the 5′ and 3′ ends of the gene and then modified these sequences to reflect preferred codon usage in *L. monocytogenes*. These 5′ and 3′ consensus oligonucleotides, 5′-GGG-[AAGCTT(*Hin*dIII)]-AAA GC(A/T)AA(C/T)GC(A/T)TATGG(A/T)CATGG-3′ and 5′-GGG-[AAGCTT (*Hin*dIII)]-GATCCAT(A/G)CAAAT(A/G)CG(A/G)CC-3′, respectively, were used as primers in a PCR using chromosomal DNA from either *L. monocytogenes* or *B. subtilis* as the template. A product of about 850 nucleotides was obtained from each. Translation of the sequenced product from the *L. monocytogenes* template showed that it resembled the alanine racemases of the gram-positive organisms.

A similar strategy was used to infer the presence and to sequence the central portion of a D-amino acid aminotransferase (*dat*) gene of *L. monocytogenes*, based on published sequences from *B. sphaericus* (12), *Bacillus* sp. strain YM-1 (45), and *Staphylococcus haemolyticus* (35). The 5′ and 3′ oligonucleotide primers were 5′-GGG-[AAGCTT(*Hin*dIII)]-GGTTATGT(A/T)TT(T/C)GGTGAT GG-3′ and 5′-GGG-[AAGCTT(*Hin*dIII)]-TTTAATATCACA(A/G)CG(T/A)A A/GCC-3′, respectively. In this case, we obtained a PCR product of about 400 nucleotides whose DNA sequence and translation showed significant homology with the aminotransferase genes of the other organisms.

Strategy for sequence determination of the complete genes. We determined the sequence of the remaining portions of the *L. monocytogenes dal* gene adjoined to the 5′ and 3′ ends of the original 850-bp PCR product by anchored PCRs (36). Briefly, this strategy used a *Bgl*II restriction digest (for the 5′ portion of the gene) or an *Xba*I digest (for the 3′ portion of the gene) of *Listeria* chromosomal DNA, onto the ends of which was then ligated a small fragment of DNA containing the T7 promoter. A 5′-portion PCR product and a 3′-portion PCR product were then synthesized and sequenced by using primers from within the central *dal* gene PCR product and a second primer homologous to the T7 promoter fragment. This procedure permitted determination of the entire sequence of the gene.

The sequence of the remainder of the *dat* gene was determined by use of an inverse PCR (8, 49). Briefly, a *Hin*dIII digest of *Listeria* chromosomal DNA was permitted to self-ligate under conditions of low DNA concentration so that mainly single circular molecules were produced. Outward-directing primers with homologies at the two ends of the original PCR segment of the gene were then used to make a new PCR product that began at the 5′ end of the original PCR segment and continued to the 5′ end of the gene, through the *Hin*dIII self-ligation site, and into the 3′ end of the gene. Using this method, we obtained the sequence of the entire gene.

Production of mutations in the *dal* and *dat* genes. The *dal* gene was initially inactivated by means of a double-allelic exchange between the chromosomal gene and the temperature-sensitive shuttle plasmid pKSV7 (42) carrying an erythromycin resistance gene (39) between a 450-bp fragment from the 5′ end of the original 850-bp *dal* gene PCR product and a 450-bp fragment from the 3′ end of the *dal* gene PCR product, following the protocol of Camilli et al. (7). Subsequently a *dal* deletion mutant covering 82% of the gene was constructed by a similar exchange reaction with pKSV7 carrying homology regions from the 5′ and 3′ ends of the intact gene (including sequences upstream and downstream of the gene) surrounding the desired deletion. PCR analysis was used to confirm the structure of this chromosomal deletion.

The chromosomal *dat* gene of *L. monocytogenes* was inactivated by a similar allelic exchange reaction. pKSV7 was modified to carry 450-bp fragments derived by PCR from both the 5′ and 3′ ends of the intact *dat* gene (including sequences upstream and downstream of the gene). These two fragments were ligated by appropriate PCR. Exchange of this construct into the chromosome resulted in the deletion of 30% of the central bases of the *dat* gene, which was confirmed by PCR analysis.

Infection of cells in culture. To examine the intracellular growth of the attenuated strain of *L. monocytogenes* in cell culture, monolayers of J774 cells, a murine macrophage-like cell line, primary murine bone marrow-derived macrophages, and the human HeLa line were grown on glass coverslips and infected as described previously (34). To enhance the efficiency of infection of HeLa cells, a naturally nonphagocytic cell line, the added bacteria were centrifuged onto the HeLa cells at 543 × *g* for 15 min. At various times after infection, samples of the cultures were taken for differential staining, for the determination of viable intracellular bacteria, or for immunohistochemical analysis.

Immunohistochemistry. Coverslips with infected macrophages or HeLa cells were washed with phosphate-buffered saline, fixed in 3.2% formalin, and permeabilized with 0.05% Tween 20. Bacteria were detected with fluorescein isothiocyanate (FITC)-labeled rabbit anti-*Listeria* antiserum (Molecular Probes, Eugene, Oreg.) or with rabbit anti-*Listeria* antiserum (Listeria O Antiserum Poly; Difco Laboratories) followed by lissamine rhodamine sulfonyl chloride (LSRSC)-labeled donkey anti-rabbit antibodies or coumarin-labeled goat anti-rabbit antibodies. Actin was detected with FITC- or tetramethylrhodamine isothiocyanate (TRITC)-labeled phalloidin. To distinguish extracellular (or phagosomal) from intracytosolic bacteria, the former were stained prior to permeabilization.

Induction of LLO-specific CTLs. Female BALB/c mice, 6 to 8 weeks of age (Charles River Laboratories, Raleigh, N.C.), were immunized by intraperitoneal inoculation with either the wild-type or *dal dat* strain of *L. monocytogenes*. After 14 days, some of the mice were boosted with a second inoculation at the same number of microorganisms. Ten or more days after the last inoculation, 6×10^7 splenocytes from a given animal were incubated in Iscove's modified Dulbecco modified Eagle medium with 3×10^7 splenocytes from that same animal that had been loaded with 10 μM LLO peptide 91-99 during a 60-min incubation at 37°C. After 5 days of in vitro stimulation, the resulting cultures were assayed for the presence of CTL activity capable of recognizing LLO peptide-labeled P815 cells as previously described (13, 53). Every determination of lytic activity was corrected for activity on unlabeled target cells, which showed between 1 and 10% lysis in different experiments.

Animal studies. Fifty percent lethal dose (LD_{50}) assays were performed by injecting female BALB/c mice (Bantin-Klingman, Freemont, Calif.) at 8 weeks of age with 0.2 ml of three- to fivefold serial dilutions of bacteria as described previously (2). The LD_{50} of wild-type *L. monocytogenes* strain 10403 in these animals is approximately 10^4. The LD_{50} of the *dal dat* double-mutant strain of *L. monocytogenes* was found to be $>8 \times 10^8$ or, when injected in the presence of 20 mg of D-alanine in the 0.2-ml injection volume, approximately 7×10^7.

To examine the protection produced by immunization with the *dal dat* mutant, groups of four to five BALB/c mice were injected with viable wild-type or *dal dat* double-mutant bacteria (in the presence or absence of 20 mg of D-alanine) by tail vein injection. Three to four weeks following immunization, the mice were challenged with approximately 10 LD_{50} of viable wild-type *L. monocytogenes* 10403 in 0.2 ml by tail vein injection. Spleens were removed 48 h later and homogenized individually in 4.5 ml of phosphate-buffered saline–1% proteose peptone in a tissue homogenizer (Tekmar). The homogenates were serially diluted and plated onto BHI agar. Log_{10} protection was determined by subtracting the mean of the log_{10} CFU/spleen values of the test group from the mean of the log_{10} CFU/spleen values of the untreated control group.

Nucleotide sequence accession numbers. The nucleotide sequences of the *L. monocytogenes dal* and *dat* genes, shown in Fig. 1 and 3, respectively, have GenBank accession no. AF038438 and AF038439.

RESULTS

Construction of a strain of *L. monocytogenes* defective in cell wall synthesis. We determined whether *L. monocytogenes* harbors genes expected to be required for the synthesis of D-alanine. The alanine racemase (*dal*) gene, used by many microorganisms for the synthesis of D-alanine, has been sequenced in *Salmonella typhimurium* (14, 51), *B. subtilis* (10), and *B. stearothermophilis* (46) but has not been found in *L. monocytogenes*. To search for evidence of the gene in this organism, we synthesized primers based on the sequences (adjusted for preferred codon usage in *L. monocytogenes*) of two highly conserved regions of the gene present in the two gram-positive organisms and used these in a PCR on *L. monocytogenes* chromosomal DNA. A product that showed significant homology with the published *dal* gene sequences was obtained. The sequence of the remainder of the *L. monocytogenes dal* gene was determined (see Materials and Methods) and is shown in Fig. 1. The translated protein sequence showed 44 to 53% identity with the alanine racemase proteins of the gram-positive microorganisms and is shown in comparison with these sequences in Fig. 2.

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1       ATGGTGACA GGCTGGCAT CGTCCAACA TGGATTGAA ATAGACCGC GCAGCAATT CGCGAAAAT ATAAAAAAT GAACAAAAT AAACTCCCG GAAAGTGTC
        M  V  T   G  W  H   R  P  T   W  I  E   I  D  R   A  A  I   R  E  N   I  K  N   E  Q  N   K  L  P   E  S  V>
100     GACTTATGG GCAGTAGTC AAAGCTAAT GCATATGGT CACGGAATT ATCGAAGTT GCTAGGACG GCGAAAGAA GCTGGAGCA AAAGGTTTC TGCGTAGCC
        D  L  W   A  V  V   K  A  N   A  Y  G   H  G  I   I  E  V   A  R  T   A  K  E   A  G  A   K  G  F   C  V  A>
199     ATTTTAGAT GAGGCACTG GCTCTTAGA GAAGCTGGA TTTCAAGAT GACTTTATT CTTGTGCTT GGTGCAACC AGAAAAGAA GATGCTAAT CTGGCAGCC
        I  L  D   E  A  L   A  L  R   E  A  G   F  Q  D   D  F  I   L  V  L   G  A  T   R  K  E   D  A  N   L  A  A>
298     AAAAACCAC ATTTCACTT ACTGTTTTT AGAGAAGAT TGGCTAGAG AATCTAACG CTAGAAGCA ACACTTCGA ATTCATTTA AAAGTAGAT AGCGGTATG
        K  N  H   I  S  L   T  V  F   R  E  D   W  L  E   N  L  T   L  E  A   T  L  R   I  H  L   K  V  D   S  G  M>
397     GGGCGTCTC GGTATTCGT ACGACTGAA GAAGCACGG CGAATTGAA GCAACCAGT ACTAATGAT CACCAATTA CAACTGGAA GGTATTTAC ACGCATTTT
        G  R  L   G  I  R   T  T  E   E  A  R   R  I  E   A  T  S   T  N  D   H  Q  L   Q  L  E   G  I  Y   T  H  F>
496     GCAACAGCC GACCAGCTA GAAACTAGT TATTTTGAA CAACAATTA GCTAAGTTC CAAACGATT TTAACGAGT TTAAAAAAA CGACCAACT TATGTTCAT
        A  T  A   D  Q  L   E  T  S   Y  F  E   Q  Q  L   A  K  F   Q  T  I   L  T  S   L  K  K   R  P  T   Y  V  H>
595     ACAGCCAAT TCAGCTGCT TCATTGTTA CAGCCACAA ATCGGGTTT GATGCGATT CGCTTTGGT ATTTCGATG TATGGATTA ACTCCCTCC ACAGAAATC
        T  A  N   S  A  A   S  L  L   Q  P  Q   I  G  F   D  A  I   R  F  G   I  S  M   Y  G  L   T  P  S   T  E  I>
694     AAAACTAGC TTGCCGTTT GAGCTTAAA CCTGCACTT GCACTCTAT ACCGAGATG GTTCATGTG AAAGAACTT GCACCAGGC GATAGCGTT AGCTACGGA
        K  T  S   L  P  F   E  L  K   P  A  L   A  L  Y   T  E  M   V  H  V   K  E  L   A  P  G   D  S  V   S  Y  G>
793     GCAACTTAT ACAGCAACA GAGCGAGAA TGGGTTGCG ACATTACCA ATTGGCTAT GCGGATGGA TTGATTCGT CATTACAGT GGTTTCCAT GTTTTAGTA
        A  T  Y   T  A  T   E  R  E   W  V  A   T  L  P   I  G  Y   A  D  G   L  I  R   H  Y  S   G  F  H   V  L  V>
892     GACGGTGAA CCAGCTCCA ATCATTGGT CGAGTTTGT ATGGATCAA ACCATCATA AAACTACCA CGTGAATTT CAAACTGGT TCAAAAGTA ACGATAATT
        D  G  E   P  A  P   I  I  G   R  V  C   M  D  Q   T  I  I   K  L  P   R  E  F   Q  T  G   S  K  V   T  I  I>
991     GGCAAAGAT CATGGTAAC ACGGTAACA GCAGATGAT GCCGCTCAA TATTTAGAT ACAATTAAT TATGAGGTA ACTTGTTTG TTAAATGAG CGCATACCT
        G  K  D   H  G  N   T  V  T   A  D  D   A  A  Q   Y  L  D   T  I  N   Y  E  V   T  C  L   L  N  E   R  I  P>
1090    AGAAAATAC ATCCATTAG
        R  K  Y   I  H  *>
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FIG. 1. Nucleotide sequence and translation of the alanine racemase (*dal*) gene of *L. monocytogenes*. The gene was inactivated either by insertion of a 1.35-kb fragment of DNA encoding erythromycin resistance at a *Spe*I site at nucleotide 517 or by deletion of nucleotides 44 to 948.

The gene was inactivated by insertion of a 1.35-kb fragment of DNA encoding erythromycin resistance at a *Spe*I site near the center of the gene. The resulting *dal* bacteria were found to grow both in rich bacteriological medium (BHI) and in a synthetic medium (48) in the presence or absence of D-alanine (not shown). A mutation of the *dal* gene constructed by an in-frame deletion covering 82% of the gene (from nucleotides 44 to 949) had the same properties.

A second enzyme used by some bacteria for synthesis of D-alanine is D-amino acid aminotransferase, encoded by the *dat* gene (12, 35, 45). Using a strategy similar to that used to detect the *dal* gene in *L. monocytogenes*, we obtained a PCR product that showed significant sequence homology with known *dat* genes and gene products. The sequence of the remainder of the *dat* gene was determined (see Materials and Methods) and is shown in Fig. 3. Its deduced protein sequence showed 49 to 51% identity with sequences of aminotransferases of other gram-positive organisms. Comparison of these *dat* gene products is shown in Fig. 4.

This gene was inactivated by in-frame deletion of 31% of its

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LMDAL      1  - MVTGWHRPTWIEIDRAAIRENLKNEQNKLPESVDLWAVVKANA  43
BSTDAL     1  - - MNDFHRDTWAEVDLDAIYDNVENLRRLLPDDTHIMAVVKANA  42
BSUBDAL    1  MSTKPFYRDTWAEIDLSAIKENVSNMKKHIGEHVHLMAVEKANA  44

LMDAL     44  YGHGIIEVARTAKEAGAKGFCVAILDEALALREAGFQDDFILVL  87
BSTDAL    43  YGHGDVQVARTALERGPPP-AVAFLDEALALREKGIEAP-ILVL  84
BSUBDAL   45  YGHGDAETAKAALDAGASCLAMAILDEAISLRKKGLKAP-ILVL  87

LMDAL     88  GATRKEDANLAAKNHISLTVFREDWLENL-TL-EA---TLRI--  124
BSTDAL    85  GASRPADAALAAQQRIALTVFRSDWLEEASALYSG---PFPIHF  125
BSUBDAL   88  GAVPPEYVAIAAEYDVTLTGYSVEWLQEA-AR-HTKKGSLL--HF  127

LMDAL    125  HLKVDSGMGRLGIRTTEEARRIEATSTNDHQLQLEGIYTHFATA  168
BSTDAL   126  HLKMDTGMGRLGVKDEEETKRIVALIERHPHFVLEGLYTHFATA  169
BSUBDAL  128  HLKVDTGMNRLGVKTEEEVQNVMAILDRNPRLKCKGVFTHFATA  171

LMDAL    169  DQLETSYFEQQLAKFQTILTSLKKRPTYVHTANSAASL-LQPQI  211
BSTDAL   170  DEVNTDYFSYQYTRFLHMLEWLPSRPPLVHCANSAASLR-FPDR  212
BSUBDAL  172  DEKERGYFLMQFERFKELIAPLPLKNLMVHCANSAAGLRLKKGF  215

LMDAL    212  GFDAIRFGISMYGLTPSTEIKTSLPFELKPALALYTEMVHVKEL  255
BSTDAL   213  TFNMVRFGIAMYGLAPSPGIKPLLPYPLKEAFSLHSRLVHVKKL  256
BSUBDAL  216  -FNAVRFGIGMYGLRPSADMSDEIPFQLRPAFTLHSTLSHVKLI  258

LMDAL    256  APGDSVSYGATYTATERFWVATLPIGYADGLIRHYSGFHVLVDG  299
BSTDAL   257  QPGEKVSYGATYTAQTEEWIGTIPIGYADG-VRRLQHFHVLVDG  299
BSUBDAL  259  RKGESVSYGAEYTAEKDTWIGTVPVGYADGWLRKLKGTDILVKG  302

LMDAL    300  EPAPIIGRVCMDQTIIKLPREFQTGSKVTIIGKDHGNTVTADDA  343
BSTDAL   300  QKAPIVGRICMDQCMIRLPGPLPVGTKVTLIGRQGDEVISIDDV  343
BSUBDAL  303  KRLKIAGRICMDQFMVELDQEYPPGTKVTLIGRQGDEYISMDEI  346

LMDAL    344  AQYLDTINYEVTCLLNERIPRKYIH                    368
BSTDAL   344  ARHLETINYEVPCTISYRVPRIFFRHKRIMEVRNAIGRGESSA  386
BSUBDAL  347  AGRLETINYEVACTISSRVPRMFLENGSIMEVRNPLLQVNISN  389
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FIG. 2. Linear alignment of deduced protein sequences of alanine racemases of *L. monocytogenes* (LMDAL), *B. stearothermophilus* (BSTDAL), and *B. subtilis* (BSUBDAL). Identical amino acids are boxed.

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1       ATGAAAGTA TTAGTAAAT AACCATTTA GTTGAAAGA GAAGATGCC ACAGTTGAC ATTGAAGAC CGCGGATAT CAGTTTGGT GATGGTGTA TATGAAGTA
        M K V   L V N   N H L   V E R   E D A   T V D   I E D   R G Y   Q F G   D G V   Y E V>
100     GTTCGTCTA TATAATGGA AAATTCTTT ACTTATAAT GAACACATT GATCGCTTA TATGCTAGT GCAGCAAAA ATTGACTTA GTTATTCCT TATTCCAAA
        V R L   Y N G   K F F   T Y N   E H I   D R L   Y A S   A A K   I D L   V I P   Y S K>
199     GAAGAGCTA CGTGAATTA CTTGAAAAA TTAGTTGCC GAAAATAAT ATCAATACA GGGAATGTC TATTTACAA GTGACTCGT GGTGTTCAA AACCCACGT
        E E L   R E L   L E K   L V A   E N N   I N T   G N V   Y L Q   V T R   G V Q   N P R>
298     AATCATGTA ATCCCTGAT GATTTCCCT CTAGAAGGC GTTTTAACA GCAGCAGCT CGTGAAGTA CCTAGAAAC GAGCGTCAA TTCGTTGAA GGTGGAACG
        N H V   I P D   D F P   L E G   V L T   A A A   R E V   P R N   E R Q   F V E   G G T>
397     GCGATTACA GAAGAAGAT GTGCGCTGG TTACGCTGT GATATTAAG AGCTTAAAC CTTTTAGGA AATATTCTA GCAAAAAAT AAAGCACAT CAACAAAAT
        A I T   E E D   V R W   L R C   D I K   S L N   L L G   N I L   A K N   K A H   Q Q N>
496     GCTTTGGAA GCTATTTTA CATCGCGGG GAACAAGTA ACAGAATGT TCTGCTTCA AACGTTTCT ATTATTAAA GATGGTGTA TTATGGACG CATGCGGCA
        A L E   A I L   H R G   E Q V   T E C   S A S   N V S   I I K   D G V   L W T   H A A>
595     GATAACTTA ATCTTAAAT GGTATCACT CGTCAAGTT ATCATTGAT GTTGCGAAA AAGAATGGC ATTCCTGTT AAAGAAGCG GATTTCACT TTAACAGAC
        D N L   I L N   G I T   R Q V   I I D   V A K   K N G   I P V   K E A   D F T   L T D>
694     CTTCGTGAA GCGGATGAA GTGTTCATT TCAAGTACA ACTATTGAA ATTACACCT ATTACGCAT ATTGACGGA GTTCAAGTA GCTGACGGA AAACGTGGA
        L R E   A D E   V F I   S S T   T I E   I T P   I T H   I D G   V Q V   A D G   K R G>
793     CCAATTACA GCGCAACTT CATCAATAT TTTGTAGAA GAAATCACT CGTGCATGT GGCGAATTA GAGTTTGCA AAATAA
        P I T   A Q L   H Q Y   F V E   E I T   R A C   G E L   E F A   K *>
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FIG. 3. Nucleotide sequence and translation of the D-amino acid aminotransferase (*dat*) gene of *L. monocytogenes*. The gene was inactivated by deletion of nucleotides 370 to 636.

central region (from nucleotides 370 to 636). The growth of the resulting *dat* bacteria in broth and synthetic media was again found to be independent of the presence of D-alanine.

A *dal dat* double-mutant strain of *L. monocytogenes* was produced by a double-allelic exchange reaction between the erythromycin-resistant *dal* organism and the shuttle vector carrying the *dat* gene deletion. The growth of the resulting double mutant in bacteriological media was found to be completely dependent on the presence of D-alanine (Fig. 5). A double mutant containing deletions in both of the genes had the same phenotype. The growth defect of the double-deletion strain in the absence of D-alanine could be complemented by a plasmid carrying the *dal* gene of *B. subtilis* (not shown). All of the experiments reported used the single-deletion strain.

Defective growth of the *dal dat* double mutant in eukaryotic cells. Our original hypothesis was that a defect in the ability of *L. monocytogenes* to synthesize D-alanine would be expressed as an inability to replicate in the cytoplasm of eukaryotic cells due to the absence of the required substrate at that site. To test this hypothesis, several cell lines and primary cells in culture were examined after infection with the wild-type and mutant strains of the organism.

J774 cells are mouse macrophage-like cells that readily take up *L. monocytogenes* by phagocytosis and permit its cytoplasmic growth following escape of the bacteria from the phagolysosome (47). Figures 6A and B show typical cells seen at 5 h after infection with wild-type *L. monocytogenes* and with the *dal dat* double mutant, respectively. Whereas large numbers of

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LMDAT      1  M-KVLVNNHLVEREDATVDIEDRGYQFGDGVYEVVRLYNGKFFT  43
SHAEDAT    1  MTKVFINGEFIDQNEAKVSYEDRGYVFGDGIYEYIRAYDGKLFT  44
BSPHDAT    1  MAYSLWNDQIVEEGSITISPEDRGYQFGDGIYEVIKVYNGHMFT  44
BSPDAT     1  MGYTLWNDQIVKDEEVKIDKEDRGYQFGDGVYEVVKVYNGEMFT  44

LMDAT     44  YNEHIDRLYASAAKIDLVIPYSKEELRELLEKLVAENNINTGNV  87
SHAEDAT   45  VTEHFERFIRSASEIQLDLGYTVEELIDVVRELLKVNNIQNGGI  88
BSPHDAT   45  AQEHIDRFYASAEKIRLVIPYTKDVLHKLLHDLIEKNNLNTGHV  88
BSPDAT    45  VNEHIDRLYASAEKIRITIPYTKDKFHQLLHELVEKNELNTGHI  88

LMDAT     88  YLQVTRGVQNPRNHVIPDDFPLEGVLTAAAREVPRNERQFVEGG  131
SHAEDAT   89  YIQATRGV-APRNHSFPTPEVKPVIMAFAKSYDRPYDDLENGI  130
BSPHDAT   89  YFQITRGT-TSRNHIFPDASVPAVLTGNVKTGERSIENFEKGV  130
BSPDAT    89  YFQVTRGT-SPRAHQFPENTVKPVIIGYTKENPRPLENLEKGV  130

LMDAT    132  TAITEEDVRWLRCDIKSLNLLGNILAKNKAHQQNALEAILHRGE  175
SHAEDAT  131  NAATVEDIRWLRCDIKSLNLLGNVLAKEYAVKYNAGEAIQHRGE  174
BSPHDAT  131  KATLVEDVRWLRCDIKSLNLLGAVLAKQEASEKGCYEAILHRGD  174
BSPDAT   131  KATFVEDIRWLRCDIKSLNLLGAVLAKQEAHEKGCYEAILHRNN  174

LMDAT    176  QVTECSASNVSIIKDGVLWTHAADNLILNGITRQVIIDVAKKNG  219
SHAEDAT  175  TVTEGASSNVYAIKDGAIYTHPVNNYILNGITRKVIKWISEDED  218
BSPHDAT  175  IITECSSANVYGIKDGKLYTHPANNYILNGITRQVILKCAAEIN  218
BSPDAT   175  TVTEGSSSNVFGIKDGILYTHPANNMILKGITRDVVIACANEIN  218

LMDAT    220  IPVKEADFTLTDLREADEVFISSTTIEITPLTHIDGVQVADGKR  263
SHAEDAT  219  IPFKEETFTVEFLKNADEVIVSSTSAEVTPVVKIDGEQVGDGKV  262
BSPHDAT  219  LPVIEEPMTKGDLLTMDEIIVSSVSSEVTPVIDVDGQQIGAGVP  262
BSPDAT   219  MPVKEIPFTTHEALKMDELFVTSTTSEITPVIEIDGKLIRDGKV  262

LMDAT    264  GPITAQLHQYFVEEITRACGELEFAK                    289
SHAEDAT  263  GPVTRQLQEGFNKYIESRSS                         282
BSPHDAT  263  GEWTRKLQKAFEAKLPISINA                        283
BSPDAT   263  GEWTRKLQKQFETKIPKPLHI                        283
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FIG. 4. Linear alignment of deduced protein sequences of D-amino acid aminotransferases of *L. monocytogenes* (LMDAT), *S. haemolyticus* (SHAEDAT), *B. sphaericus* (BSPHDAT), and *Bacillus* sp. strain YM-1 (BSPDAT). Identical amino acids are boxed.

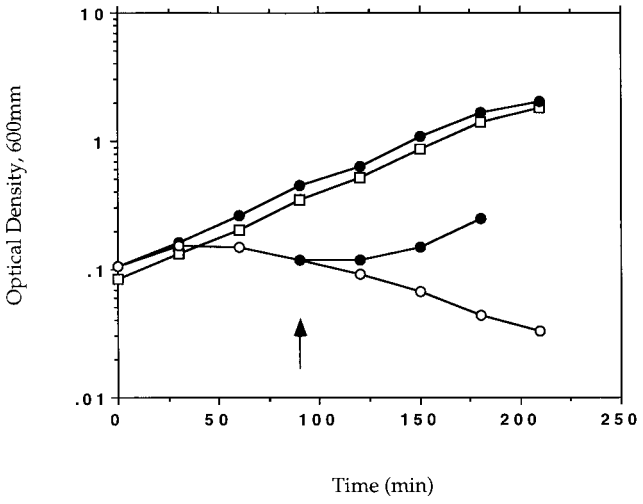

FIG. 5. Growth requirement for D-alanine of the *dal dat* double-mutant strain of *L. monocytogenes*. The *dal dat* (○, ●) and wild-type (□) strains of *L. monocytogenes* were grown in liquid culture (BHI medium) in the presence (●) or absence (○, □) of exogenous D-alanine (100 μg/ml) at 37°C. An aliquot of the mutant culture was provided D-alanine at 90 min. The starting cultures were in log phase of growth. As D-alanine had no effect on the growth of wild-type *L. monocytogenes*, those data are not shown.

bacteria were associated with those mouse cells infected with the wild-type strain, few bacteria could be found in any cells following infection with the double mutant. Infection by double-mutant bacteria in culture medium containing D-alanine permitted bacterial growth indistinguishable from that seen after wild-type infection (Fig. 6C).

The number of intracellular bacteria (defined by gentamicin resistance) that could form colonies on medium containing D-alanine was determined at several times after infection (at a multiplicity of infection [MOI] of about 0.05 bacteria per mouse cell) (Fig. 7A). The data clearly show that the double mutant was unable to replicate in J774 cells and in fact slowly died during the course of the experiment. Figure 7A also shows that the replication-defective phenotype of the double mutant could be suppressed by the inclusion of D-alanine (at 100 μg/ml) in the tissue culture medium at the time of infection and that the suppression was reversed 2 h after removal of the D-alanine. We also examined the phenotype of the mutant bacteria in primary mouse bone marrow-derived macrophages and in the HeLa line of human epithelial cells and found that the double mutant was unable to replicate in either of those cell types as well (Fig. 7B and C).

Within a few hours after infection of cells by *L. monocytogenes* when the bacteria have escaped from the phagosome, host actin filaments form a dense cloud around intracytosolic bacteria and then rearrange to form a polarized tail which propels the bacteria through the cytoplasm (9, 47). The bacterium-associated actin can readily be visualized by fluorescence-tagged phalloidin, while total bacteria can be detected with appropriately labelled anti-*Listeria* antibodies. To determine the intracytoplasmic status of the double-mutant bacteria following infection, we examined the distribution of cytoplasmic actin in the infected cells.

As shown in Fig. 8A and Table 1, at 2 h after infection (at an MOI of about 5 bacteria per cell), we found that 25.2% of wild-type bacteria associated with J774 macrophages were surrounded with a halo of stained actin and therefore were intracytosolic. By 5 h, 100% showed actin staining, some with long actin tails (Fig. 8B). However, the staining of actin in double-mutant-infected macrophages was much rarer (less than 2%). Nevertheless, if D-alanine was present during only the 30-min period of bacterial adsorption, at 2 h after infection 22% of the cell-associated mutant bacteria were surrounded with actin (Fig. 8C). At 5 h (D-alanine absent from 0.5 to 5 h), the number of intracytosolic bacteria was still only 26.7% (Fig. 8D). This indicated that few additional bacteria had entered the cytosol after removal of the D-alanine and that any bacteria already present in the cytosol had not replicated. If D-alanine was present during the entire infection (Fig. 8E), the results at 2 and 5 h were virtually indistinguishable from the wild-type infection.

Since J774 cells have been culture adapted and reflect few of the normal properties of tissue macrophages, we examined the entry of the mutant bacteria into the cytosol of primary bone marrow-derived macrophages which had been in culture for only 6 days. Because these cells show significant bacterial killing capacity, they were infected at a ratio of about 50 bacteria per cell. In this experiment, at 2 h after infection, 6.8% of the double-mutant bacteria were found to be associated with actin, and this number increased to the level seen after wild-type infection (20%) by inclusion of D-alanine during the first 30 min of the infection (18.2%) or during the entire 2-h infection (19.4%). Therefore, depending on the cell type examined, mu-

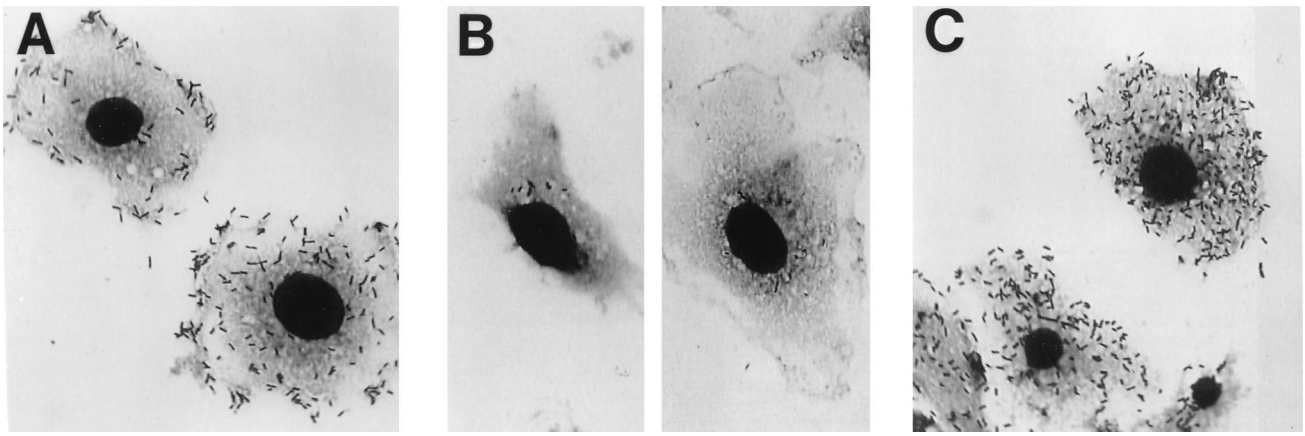

FIG. 6. Light micrographs showing the growth of wild-type (A) and *dal dat* double-mutant (B) strains of *L. monocytogenes* in J774 macrophages at 5 h after infection (at approximately 5 bacteria per mouse cell). (C) Infection by double-mutant bacteria in the continuous presence of D-alanine (80 μg/ml).

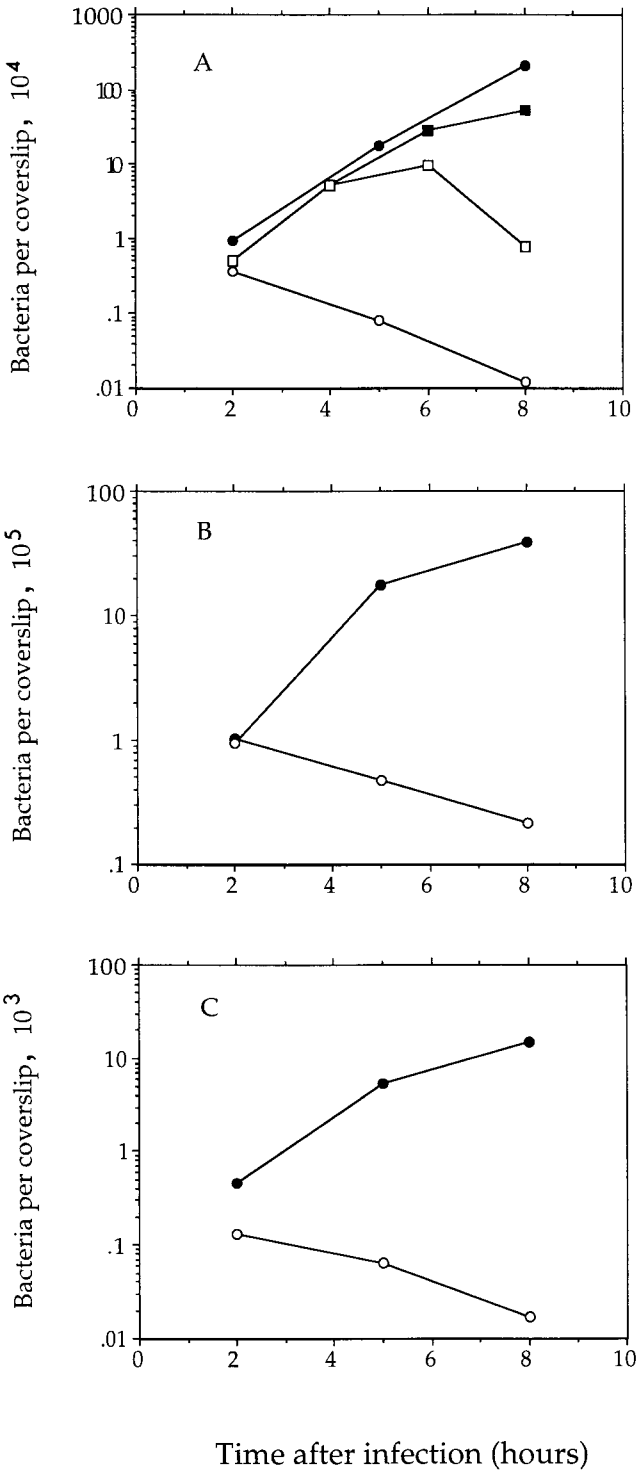

FIG. 7. Infection of mammalian cells with the *dal dat* double-mutant (○) and wild-type strains of *L. monocytogenes* (●). (A) J774 murine macrophage-like cells (MOI of about 0.05). Mutant infection in one culture (■) was in the continuous presence of D-alanine (100 μg/ml); cells in an aliquot of that culture (□) were resuspended in D-alanine-free medium at 4 h. (B) Primary murine bone marrow-derived macrophages (MOI of about 5). (C) Human epithelial cells (HeLa) (MOI of about 5). Starting cultures of *L. monocytogenes* were in stationary phase of growth.

tant bacteria in the absence of D-alanine had either a low or a moderate efficiency of entering the host cytosol (or, alternatively, showed reduced binding of actin onto their surface). However, the brief presence of D-alanine during the initial phase of infection allowed a normal fraction of bacteria to enter the cytosol and bind actin.

In vivo attenuation of the *dal dat* double mutant. The LD_{50} of wild-type *L. monocytogenes* in BALB/c mice is approximately 10^4 (2). To determine the virulence of the *dal dat* double-mutant strain relative to wild-type bacteria, groups of mice were injected with graded doses of the mutant organism. The LD_{50} of the double mutant was found to be greater than 8×10^8, indicating that this strain was highly attenuated. When the inoculum in these experiments contained D-alanine (20 mg), the LD_{50} was found to be lowered 10-fold to approximately 7×10^7.

After sublethal infection of mice with wild-type *L. monocytogenes*, the bacteria survive and replicate in the spleens and livers of infected animals for up to 5 to 7 days, followed by the onset of a sterilizing immunity. The extent of the in vivo persistence of the mutant bacteria in the spleens of infected animals was therefore examined following infection with 2×10^7 mutant bacteria and compared with the result of infection by 4×10^2 wild-type organisms. The results in Fig. 9 show that whereas this low dose of wild-type *L. monocytogenes* resulted in a peak of replication at 2 to 3 days, increasing the number of bacteria in this organ by several logs, the mutant bacteria fell to almost undetectable levels within 2 days. The presence of D-alanine in the inoculum allowed a small number of organisms to survive somewhat longer.

Induction of an immune response with the *dal dat* double mutant. Infection of mice by *L. monocytogenes* produces a long-lived state of specific immunologic memory that enables the infected host to resist lethal challenge by the same organism for months after the primary infection. We determined whether infection of mice with sublethal doses of the *dal dat* bacteria could induce this important long-lasting state of protective immunity. Mice were injected intravenously with 2×10^7 double-mutant bacteria and challenged 3 to 4 weeks later with 10 LD_{50} of wild-type *L. monocytogenes*. D-Alanine (20 mg) was provided in the initial inoculum of mutant organisms to be certain that the organisms were fully viable at the time of initial infection. The data in Fig. 10 shows that following a single infection with the mutant bacteria, the level of antilisterial protection was approximately 3 $\log_{10}$, similar to the protection generated by immunization with 4×10^2 wild-type organisms. Infection with 2×10^7 mutant bacteria without D-alanine provided little protection. The almost complete protection obtained with mutant bacteria occurred despite the fact that by 2 days postinfection more than 100-fold-fewer bacteria were detected in the spleens of mutant-infected mice than in animals infected with wild-type organisms (Fig. 9).

LLO peptide 91-99 is the major epitope of the LLO protein and one of the major epitopes to which mice respond when mounting a cell-mediated immune response against *L. monocytogenes* infection (4, 17, 31). To determine whether the protective immunity generated by infection with the attenuated *dal dat* bacteria was associated with the induction of cytolytic T cells, splenocytes from infected animals were assayed for the ability to lyse target cells loaded with this peptide. Figure 11B shows that animals that had been infected intraperitoneally with 3×10^7 double-mutant bacteria and provided D-alanine subcutaneously (40 mg) before and after the infection showed strong CTL responses directed against the LLO peptide. Likewise, mice provided with D-alanine in their drinking water (0.2 or 2 mg/ml) before and after infection mounted a modest CTL

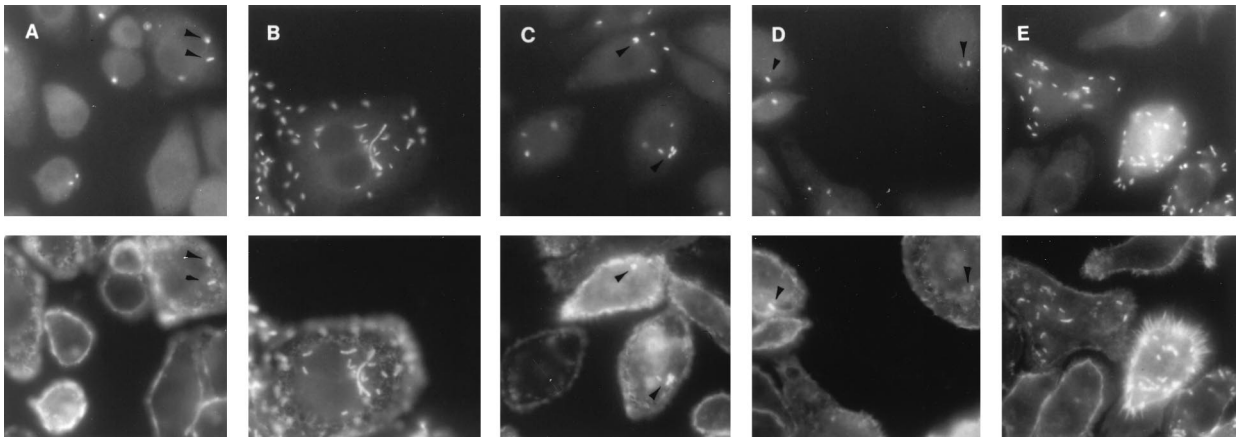

FIG. 8. Association of actin with intracytosolic wild-type *L. monocytogenes* (A, 2 h; B, 5 h) or with the *dal dat* double mutant (C, 2 h with D-alanine [100 μg/ml] present from 0 to 30 min; D, 5 h with D-alanine present from 0 to 30 min; E, 5 h with D-alanine present continuously) following infection of J774 cells. Photomicrographs in the top row show the binding of FITC-labeled antilisterial antibodies to total bacteria; those below show the binding of TRITC-labeled phalloidin to actin. Arrowheads indicate some actin-associated bacteria in the sparsely infected cells.

response after single infection with 3×10^7 mutant bacteria. In the absence of D-alanine, animals infected and boosted one time with 3×10^7 double-mutant bacteria, or animals singly infected with 3×10^8 bacteria (data not shown), also showed a modest CTL response to LLO peptide 91-99. Single infection with 3×10^7 double-mutant bacteria in the absence of D-alanine produced no significant response (Fig. 11B).

DISCUSSION

L. monocytogenes is a gram-positive facultative intracellular bacterium which has been used for decades as a model for the study of cell-mediated immunity (6, 26, 27). Long-term resistance to infection by this microorganism resides primarily in the MHC class I-restricted $CD8^+$ T cells that are induced following primary infection (3, 17). These cells act by directly lysing antigen-expressing target cells, as well as through the action of the cytokines gamma interferon and tumor necrosis factor alpha (18). We and other investigators have been exploring the use of recombinant forms of *L. monocytogenes* as a vehicle for the delivery of foreign antigens into the MHC class I pathway of antigen presentation (13, 15, 22, 32, 33, 40).

This strategy for vaccine development suffers, however, from the known pathogenicity of the organism, which is the cause of listeriosis, a food-borne disease characterized by meningitis, septicemia, abortion, and often a high rate of mortality. We therefore attempted to develop a suitably attenuated form of *L. monocytogenes* that could be used as a safe vaccine and adjuvant. Since virtually all bacterial species contain cell wall components that are unique to these organisms, we deduced that a strain of *L. monocytogenes* unable to synthesize one of these components, D-alanine (21, 43), would be crippled unless specifically supplied with this substrate.

D-Alanine appears to be synthesized by different pathways in different organisms. *E. coli* and *S. typhimurium* possess two weakly homologous alanine racemases, one constitutive and

TABLE 1. Intracytoplasmic status of bacteria following infection of J774 cells and bone marrow-derived macrophages

Cells	% Intracytosolic bacteria[a]	
	2 h	5 h
J774		
L. monocytogenes wild type	25.2 (35.6)	100 (100)
dal dat mutant		
5 h D-ala[b]	22.7 (44.4)	95 (95)
30 min D-ala[c]	22.0 (37.5)	26.7 (47.6)
No D-ala	<2 (<5)	<2 (<5)
Bone marrow macrophages		
L. monocytogenes$^+$ wild type	20.0 (68.0)	75.8 (77.8)
dal dat mutant		
5 h D-ala	19.4 (65.4)	75.0 (80.0)
30 min D-ala	18.2 (52.9)	24.8 (46.4)
No D-ala	6.8 (36.4)	13.6 (40.6)

[a] Percentage of total cell-associated bacteria that are intracytosolic, as defined by their staining for bacteria-associated actin. Numbers in parentheses represent percentages of infected macrophages containing intracytosolic, actin-associated bacteria.

[b] D-Alanine (100 μg/ml) was present throughout the infection.

[c] D-Alanine (100 μg/ml) was present only during the first 30 min of infection.

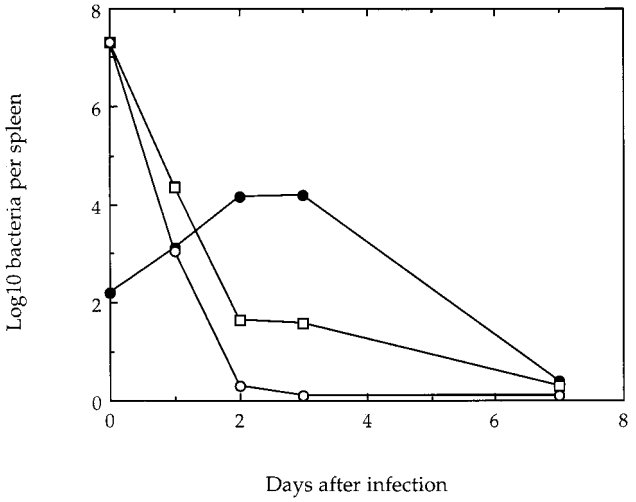

FIG. 9. Recovery of bacteria from spleens of BALB/c mice following sublethal infection with wild-type *L. monocytogenes* (●), the *dal dat* mutant in the absence of D-alanine (○), and the *dal dat* mutant in the presence of 20 mg of D-alanine in the inoculation fluid (□). The points at day 0 show the total number of viable organisms injected, not bacteria per spleen.

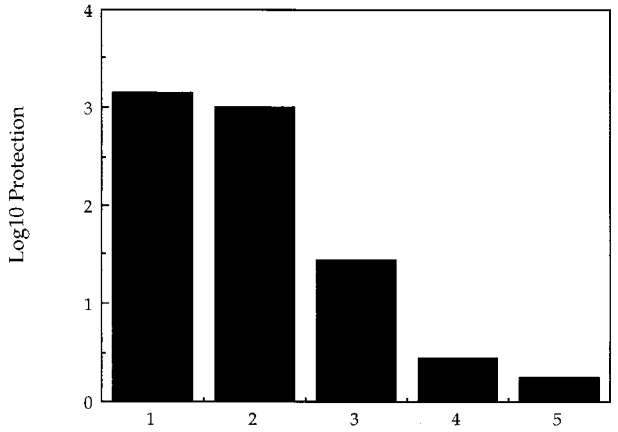

FIG. 10. Protection of BALB/c mice against challenge with $10 \times LD_{50}$ of wild-type *L. monocytogenes* by immunization with the *dal dat* double-mutant strain of *L. monocytogenes*. Column numbers represent groups of five mice immunized with the following organisms: 1, 4×10^2 CFU of wild-type *L. monocytogenes*; 2, 2×10^7 CFU of *dal dat* mutant (plus 20 mg of D-ala), 3, 2×10^5 CFU of *dal dat* mutant (plus D-ala); 4, 2×10^4 CFU of *dal dat* mutant (plus D-ala), 5, 2×10^7 CFU of *dal dat* mutant (no D-ala). Mice were challenged 21 to 28 days later. Log_{10} protection was calculated as described in Materials and Methods. The largest error seen in all mouse groups was 0.17 log_{10}.

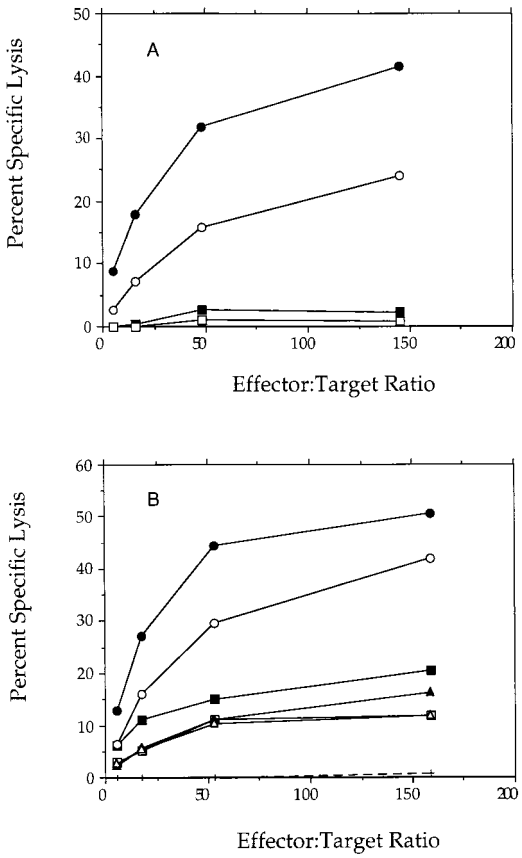

FIG. 11. Cytolytic activity of splenocytes isolated from mice 10 to 14 days after infection with wild-type *L. monocytogenes* (●, ○) or naive controls (■, □) (A). Open and closed symbols represent independent experiments; D-alanine was not provided in either experiment. (B) *dal dat* double mutant: 3×10^7 bacteria (+), 3×10^7 bacteria with boost at 10 days (▲, △); 3×10^7 bacteria with animals provided D-alanine subcutaneously (40 mg at −6 h, time of infection, 6 h, and 12 h) (●, ○), 3×10^7 bacteria with D-alanine (2 mg/ml; ■) or D-alanine (0.2 mg/ml; □) in drinking water from 24 h before infection to 36 h post-infection. Open and closed symbols represent independent experiments.

one inducible, which can convert L-alanine to D-alanine. D-Alanine produced by the first enzyme is apparently used for peptidoglycan formation, while the latter enzyme may be utilized to provide substrate to a D-alanine dehydrogenase that converts the compound to pyruvate and ammonia (50, 52). In *S. typhimurium*, a mutation in either gene alone permits the synthesis of sufficient D-alanine for cell growth, while a double mutant displays the expected phenotype of an exogenous D-alanine requirement (50). In two *Bacillus* species, alanine racemases have also been identified (10, 46). Mutation of this gene in *B. subtilis* leads to a D-alanine requirement only when the bacteria are grown in rich broth or in synthetic media that contain L-alanine (10). A second enzyme, D-amino acid aminotransferase, that can convert D-glutamic acid and pyruvate to α-ketoglutarate and D-alanine has also been identified in several gram-positive species and could be a source of D-alanine (12, 35, 45). Indeed, we have found that in *L. monocytogenes*, both a racemase gene and an aminotransferase gene are present, and both genes must be inactivated in order to produce a requirement for exogenous D-alanine.

The *dal dat* double mutant was found to be unable to replicate in bacteriological culture media devoid of added D-alanine. It was also unable to replicate following infection of several different lines of eukaryotic cells growing in standard tissue culture media. On infection of BALB/c mice with 10^7 of these bacteria, few organisms survived for longer than 1 to 2 days. Consequently, the *dal dat* strain was completely attenuated in BALB/c mice and showed an LD_{50} in these animals of $>8 \times 10^8$ (compared with 10^4 for the wild-type organism). These results support the view that if any D-alanine is present in eukaryotic cells and in mice, the levels are below the threshold required for growth of the D-alanine-requiring strain of *Listeria*.

Can this attenuated strain of *L. monocytogenes* induce an immune response in mice? When ~0.1 LD_{50} of the double mutant was administered intravenously in the absence of D-alanine, little protection against a lethal challenge by the wild-type organism was obtained. Likewise, this dose of the double mutant (given intraperitoneally) produced no detectable *Listeria*-specific CTL response. A booster infection of mice produced a modest increase in the *Listeria*-specific CTL response, as did single immunization with a higher dose of the mutant organisms. These weak responses were not surprising in view of the complete absence of any replication of these bacteria in eukaryotic cells. Indeed, the lack of D-alanine might in addition cause down-regulation of the synthesis of enzymes necessary for entry of the microorganism to the host cytosol, the minimum requirement for induction of a cell-mediated immune response. Our assays for intracytosolic bacteria (by the detection of binding of cytoplasmic actin) suggested that their entry to the cytosol was far less efficient than for wild-type *L. monocytogenes*.

However, the presence of D-alanine at the time of infection of cells in culture allowed the normal number of the double-mutant bacteria to enter the cytosol of infected cells. Additionally, we showed that eukaryotic cells were able to take up sufficient D-alanine from their growth medium to satisfy the growth requirement of the double-mutant bacteria. This uptake was reversible, so that when D-alanine was subsequently removed from the medium, the attenuation phenotype returned within several hours. It seemed possible that a similar paradigm could function in vivo to produce a novel means of in

vivo suppression of the attenuating double mutation, resulting in both entry to the cytosol and transient survival and replication of the organism to generate a moderate to strong immune response. In vertebrates, D-amino acids, which originate almost exclusively from intestinal and food bacteria, are efficiently removed from the system by the action of D-amino acid oxidases (16, 20, 25, 29).

Indeed, intravenous inoculation of these organisms (at ~0.1 LD_{50} in the presence of D-alanine) led to virtually full protection against a lethal challenge by wild-type organisms. Likewise, transient suppression of the defective phenotype of the double mutant by providing the host with D-alanine by subcutaneous injection or by inclusion in the drinking water at the time of intraperitoneal infection produced moderate or strong CTL responses against the major *L. monocytogenes* T-cell epitope, LLO peptide 91-99. Transient suppression of the attenuation thus appears to be an effective mechanism to produce a good immune response to these organisms.

The strong response seen in the protection experiment suggests that protective immunity is adequately stimulated within the first 1 or 2 days of infection, since after this time the bulk of the attenuated bacteria are gone. This result appears to differ from an early observation that showed that ampicillin administered 24 h after *L. monocytogenes* infection greatly reduced protection against subsequent challenge (30). A significant difference between these two experiments is the larger mass of antigen administered in 0.1 LD_{50} of our attenuated strain.

Other modifications of *L. monocytogenes* have been suggested for use as vaccine vectors. Mutations in the *hly* gene produce a defective hemolysin and prevent the ingested organism from escaping into the host cytosol. Such mutants can be completely avirulent (28), but they fail to present antigens to $CD8^+$ T cells (6) and therefore are a poor choice of vector for potential induction of CTL responses. It has been reported that heat-killed *L. monocytogenes*, which also should fail to enter the cytosol of infected cells, was able to induce protective $CD8^+$ T lymphocytes under appropriate circumstances. However, protection was short-lived (44). *act*A mutants are able to grow in the cytoplasm of infected cells but, because they fail to nucleate host actin, are unable to propagate the infection through cell-to-cell spread. These bacteria are capable of inducing effective CTL responses (15). However, such mutants are still virulent and persist for up to 7 days in the livers of infected mice. They also grow at normal rates in standard media; such growth represents a continuing source of bacteria that might through various genetic mechanisms become altered to regain greater virulence or even, unreverted, show virulence under unanticipated circumstances.

The hyperattenuated *dal dat* strain of *L. monocytogenes* described in this report, in which the pathways for synthesis of D-alanine have been abolished, appears to provide good immunogenicity while being unable to replicate in infected animals or in usual media and thus may represent a useful tool as a safe vaccine and adjuvant. The use of cell wall auxotrophy, as exploited here, has been explored previously as a mechanism for attenuation of virulence in *Shigella* (41).

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