INFECTION AND IMMUNITY, Nov. 1993, p. 4848–4856
0019-9567/93/114848-09$02.00/0

Vol. 61, No. 11

# Compartmentalization of Defined Epitopes Expressed in *Escherichia coli* Has Only a Minor Influence on Efficiency of Phagocytic Processing for Presentation by Class I and Class II Major Histocompatibility Complex Molecules to T Cells

MARY JO WICK,[1]†* JOHN D. PFEIFER,[1,2] KIRK A. FINDLAY,[2]‡ CLIFFORD V. HARDING,[2]‡ AND STAFFAN J. NORMARK[1]§

*Departments of Molecular Microbiology[1] and Pathology,[2] Washington University School of Medicine, St. Louis, Missouri 63110*

Received 7 July 1993/Returned for modification 9 August 1993/Accepted 21 August 1993

**The effect of abundance and compartmentalization of antigenic epitopes expressed in *Escherichia coli* on phagocytic processing was studied by expressing fusion proteins containing the epitope from positions 52 to 61 of hen egg white lysozyme [HEL(52–61)], which binds the I-A$^k$ murine major histocompatibility complex class II (MHC-II) molecule or the epitope from positions 257 to 264 of chicken egg ovalbumin [OVA(257–264)], which binds the K$^b$ murine MHC-I molecule. Epitopes expressed as fusion proteins in the outer membrane protein LamB allowed exposure of the epitopes either at the bacterial surface, in the periplasmic space, or in the cytoplasm. Regardless of epitope compartmentalization within the bacterium, MHC-II-restricted or MHC-I-restricted presentation to T hybridoma cells occurred after macrophages phagocytosed bacteria producing the HEL(52–61) epitope or the OVA(257–264) epitope, respectively. Increased epitope abundance within a given microbial compartment resulted in increased processing and presentation to epitope-specific T hybridoma cells. Minor differences in the efficiency of epitope processing between the constructs was observed, and the HEL or OVA epitope exposed in the periplasmic space was processed most efficiently compared with the surface- or cytoplasm-localized epitopes. These differences could be overcome by increasing the amount of epitope per bacterium as little as two to five times. The minor differences in processing efficiency may be due to differing protein contexts of the epitope as well as differing epitope compartmentalizations within the bacteria. Thus, production of abundant epitope is the important parameter influencing processing of epitopes expressed in *E. coli* to induce T-cell responses rather than targeting of an epitope to a specific bacterial compartment.**

Recombinant vaccines provide a potentially powerful means of immunizing with a genetically well-characterized attenuated microbe that produces defined protein(s) or peptide(s) in combinations that can result in immune responses to provide safe and effective protection to one or several pathogens simultaneously. For example, recombinant *Escherichia coli* or *Salmonella* species have been used to express several foreign proteins or epitopes derived from bacterial (14, 16, 27, 34, 40, 44) viral (2, 12, 32, 33, 41, 47), or parasitic (1) virulence-associated proteins. Recently, development of recombinant *E. coli* expressing various human immunodeficiency virus type 1 glycoproteins has been explored in the development of a vaccine for this serious pathogen (9).

Reports on the immune response with recombinant *E. coli* vaccines have provided data with respect to the humoral and cellular responses to the expressed epitopes (9, 12, 32, 33, 41, 47). The responses are reportedly influenced by parameters of the host bacterium, including the location of the epitope within the host bacterium, the route of immunization, and the level of epitope expression (32, 33, 41, 47). Recombinant vaccine strains are often constructed such that the epitope of interest is expressed on the surface of the microbe, a location which may provide a highly accessible epitope to the immune system (2, 8, 9, 12, 32, 40, 47). However, surface expression of epitopes is not required to achieve an epitope-specific immune response (1, 33, 41), and the question of epitope compartmentalization in the vaccine strain has not been systematically addressed to establish whether epitopes expressed on the bacterial surface provide any advantage for an epitope-specific immune response.

Recently, we described an in vitro murine model system to study the processing of antigenic epitopes expressed in bacteria for subsequent presentation to T cells via either major histocompatibility complex class II (MHC-II) or MHC-I molecules (42, 43). This system utilizes sequences from hen egg white lysozyme that contain the I-A$^k$-restricted epitope from positions 52 to 61 [HEL(52–61)] (39) to examine phagocytic processing of antigenic epitopes for MHC-II presentation to T cells. We have also recently described a novel phagocytic processing pathway for presentation via MHC-I molecules (42) which we have studied using the epitope from positions 257 to 264 from chicken egg ovalbumin [OVA(257–264)] that binds H-2K$^b$ (19). Our approach has been to express these defined T-cell epitopes as fusion proteins in *E. coli* and expose these bacteria to phagocytic antigen-processing cells that process and present the epitopes to T hybridoma cells specific for the peptide-MHC complex (42, 43).

Here, we use this model system to analyze the effect of

* Corresponding author. Electronic mail address: Mary_Jo.Wick@immuno.lu.se.

† Present address: The Immunology Unit, Lund University, Sölvegatan 21, 223 62 Lund, Sweden.

‡ Present address: Institute of Pathology, Case Western Reserve University, Cleveland, OH 44106.

§ Present address: Department of Bacteriology, Karolinska Institute, 10401 Stockholm, Sweden.

TABLE 1. Plasmids used in this study

| Plasmid | Description (reference) | Protein encoded | Epitope localization |
|---|---|---|---|
| pTrc99A | Cloning vector (3) | None | |
| pUHE21-2 | Expression vector (31) | None | |
| pLamBSma153 | pTrc99A containing LamB with a *Sma*I site that restricts between residues 153 and 154 | LamB | |
| pLamBHEL153 | pUHE21-2 containing LamB with the HEL(45–62) epitope inserted between residues 153 and 154 | LamBHEL153 | Surface |
| pLamBOVA153 | pUHE21-2 containing LamB with the OVA(256–265) epitope inserted between residues 153 and 154 | LamBOVA153 | Surface |
| pLamBSma183 | pTrc99A containing LamB with a SmaI site that restricts between residues 183 and 184 | LamB | |
| pLamBHEL183 | pUHE21-2 containing LamB with the HEL(45–62) epitope inserted between residues 183 and 184 | LamBHEL183 | Periplasm |
| pLamBOVA183 | pUHE21-2 containing the OVA(256–265) epitope inserted between residues 183 and 184 | LamBOVA183 | Periplasm |
| pMalEHEL | pUHE21-2 containing LamB residues 143 to 165 [inclusive of the HEL(45–62) epitope between residues 153 and 154] fused to the carboxy terminus of mature MalE | MalEHEL | Cytoplasm |
| pMalEOVA | pUHE21-2 containing LamB residues 143 to 165 [inclusive of the OVA(256–265) epitope between residues 153 and 154] fused to the carboxy terminus of mature MalE | MalEOVA | Cytoplasm |
| pJLP-1E | pUC18 containing HEL(45–61) fused to the carboxy terminus of the Crl protein (43) | Crl-HEL | Cytoplasm |
| pCrl-OVA | pUC18 containing OVA(257–277) fused to the carboxy terminus of the Crl protein (42) | Crl-OVA | Cytoplasm |

epitope abundance and compartmentalization within the microbe on phagocytic antigen processing by constructing several inducible fusion proteins that specifically express the epitopes in a defined bacterial compartment (exposed on the bacterial surface, facing the periplasmic space, or in the cytoplasm). The data show that for both MHC-I and MHC-II presentation, differences in the bacterial compartmentalization of an epitope led to only minor differences in the efficiency of processing. Epitope abundance, regardless of compartmentalization within the bacteria, strongly correlated with the level of presentation. These results suggest that the important parameter for epitope expression in recombinant vaccine strains to induce T-cell responses is abundance rather than localization in a specific bacterial compartment.

## MATERIALS AND METHODS

**Periplasm- and surface-localized epitopes: LamB fusion proteins.** Fusion proteins between the *E. coli* LamB protein and the HEL(45–62) or OVA(256–265) epitopes were constructed by polymerase chain reaction (PCR) amplification of the entire *lamB* gene from the chromosome of *E. coli* YMEL (45) with oligonucleotide primers designed from the published *lamB* sequence (13). The PCR strategy created an *Eco*RI site 54 bp 5′ to the LamB ATG, a *Bam*HI site 189 bp 3′ to the LamB stop codon, and either a *Sma*I site that restricts between codons 153 and 154 or a *Sma*I site that restricts between codons 183 and 184 (the native *Sma*I site that restricts within codon 183 was destroyed in this process). In a two-step ligation procedure, the entire coding region of *lamB* (1,585 bp) was inserted into the *Eco*RI and *Bam*HI sites of pTrc99A (3). This created two plasmids, pLamBSma153 and pLamBSma183, that contained a single *Sma*I restriction site in *lamB* after the codon for residue 153 or after residue 183, respectively (Table 1).

DNA encoding the HEL(45–62) epitope was obtained by PCR amplification of the cloned chicken egg white lysozyme gene (28). The PCR strategy used oligonucleotide primers designed from the published HEL sequence (28) to amplify a 77-bp fragment of HEL DNA containing the (45–62) epitope and incorporated a *Nru*I site that restricts between codons 44 and 45 of mature HEL and a *Bal*I site that restricts between codons 62 and 63 of mature HEL. This strategy created no amino acid substitutions within the (45–62) epitope. After restriction endonuclease digestion with *Nru*I and *Bal*I, this DNA fragment was ligated into *Sma*I-digested pLamBSma153 or pLamBSma183. Transformants were screened for the *hel* insert in the proper orientation by PCR, and a single unaltered in-frame insertion of the epitope in each fusion protein was confirmed by DNA sequence analysis (Fig. 1). The DNA encoding these two fusion proteins was cloned into the *Eco*RI-*Bam*HI sites of pUHE21-2 (31), which was modified by insertion of the *lacI*$^q$ gene (6). These plasmids, pLamBHEL153 and pLamBHEL183, produced surface- or periplasm-exposed epitope, respectively (Table 1).

DNA encoding the OVA(256–265) epitope was constructed by synthesizing complementary overlapping 30-mer oligonucleotides that exactly corresponded to the nucleotide sequence of ovalbumin residues 256 to 265 (35). The oligonucleotides were 5′ phosphorylated with T4 polynucleotide kinase and were annealed by heating to 72°C and slowly cooling to room temperature. The phosphorylated, annealed oligonucleotide pair was then ligated into *Sma*I-digested pLamBSma153 and pLamBSma183 DNAs. Transformants were screened for the *ova* insert by PCR, and nucleotide sequence analysis confirmed a single unaltered in-frame insertion of the OVA(256–265) epitope in each fusion protein (Fig. 1). The DNAs encoding LamBOVA153 and LamB OVA183 fusion proteins were subsequently cloned into the *Eco*RI-*Bam*HI sites of the expression vector pUHE21-2. The resultant plasmids, pLamBOVA153 and pLamBOVA 183, produced surface- or periplasm-exposed epitopes, respectively (Table 1). All four fusion proteins were expressed from their respective plasmids in the *lamB* deletion strain *E. coli* RAM191 (37) upon induction with isopropyl-β-D-thiogalactopyranoside (IPTG) as described below.

**Cytoplasm-localized epitopes: MalE fusion proteins.** Soluble cytoplasmic HEL and OVA fusion proteins were made by fusing the portion of LamB that already contained the

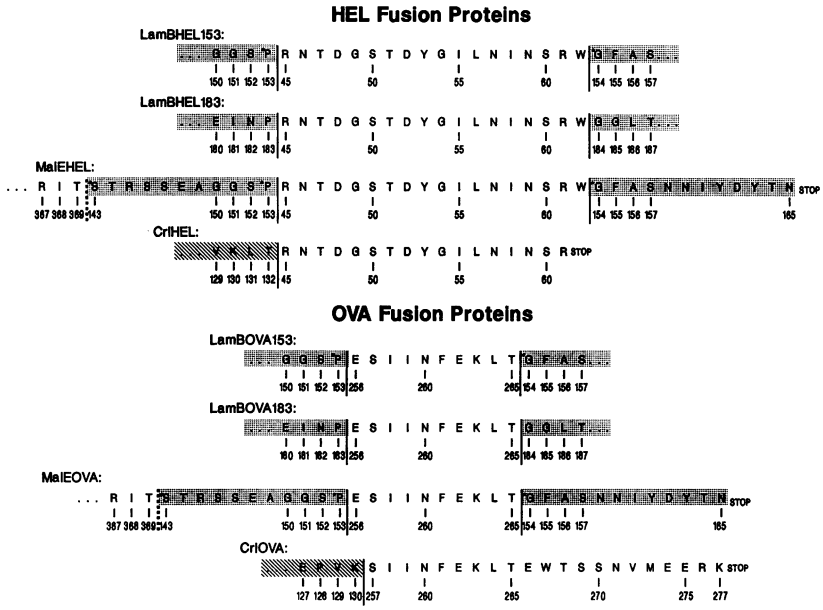


FIG. 1. Junction regions of HEL and OVA fusion proteins. The regions between the vertical bars represent the HEL or the OVA epitope in the fusion proteins. The amino acids listed within the shaded bars are residues from LamB, in which the ellipses indicate that the LamB residues before and after the epitope insertion remain unchanged from those of native LamB. For the MalE fusion proteins, the residues to the left of the dashed vertical bars indicate the carboxy-terminal three residues of mature MalE. The stop codon after residue 369 of MalE was destroyed during cloning to allow insertion of the residues from LamBHEL153 or LamBOVA153 and translational read-through. The PCR cloning strategy used to construct the MalE fusion proteins also inserted a stop codon after amino acid 165 of LamBHEL153 or LamBOVA153, as indicated, to terminate translation. For the Crl-HEL and Crl-OVA fusion proteins, the carboxyl-terminal residues from the Crl protein are indicated to the left of the vertical bar in the boxes with slashes. Translational stops are indicated. Asterisks indicate amino acid changes introduced by cloning.

HEL or OVA epitope inserted between residues 153 and 154 with the carboxy terminus of the mature *E. coli malE* gene as follows. PCR was used to amplify DNA encoding LamB amino acids 143 to 165 from either pLamBHEL153 or pLamBOVA153, constructs which already contained the HEL(45–62) or the OVA(256–265) epitope, respectively. This PCR strategy used oligonucleotide primers within LamB to create a *Sal*I site 5′ of the epitope and a *Hin*dIII site and a translational stop codon 3′ of the epitope. This 131-bp fragment was then digested with *Sal*I and *Hin*dIII and was cloned into the corresponding sites of pUHE21-2. Next, DNA encoding *malE* (17) lacking the signal sequence was PCR amplified from *E. coli* MC4100 (7) with oligonucleotide primers that created a *Bam*HI site 5′ of the first codon for the mature protein and a *Sal*I site at the end of the gene. This strategy destroyed the native stop codon of MalE to allow translational read-through of the LamBHEL153- and the LamBOVA153-coding sequences. After restriction endonuclease digestion, the *Bam*HI-*Sal*I fragment encoding mature MalE was ligated into pUHE21-2 containing the 131-bp *lamBHEL(45–62)* or *lamBOVA(256–265) Sal*I-*Hin*dIII fragment. Thus, trihybrid proteins of MalE-LamB-HEL153 (named MalEHEL) or MalE-LamB-OVA153 (named MalE OVA) (Table 1) that lacked the MalE signal sequence and were consequently localized in the bacterial cytoplasm were constructed (4).

**Growth and induction of *E. coli* expressing the fusion proteins.** *E. coli* RAM191 containing vector alone (pUHE21-2) or DNA encoding the LamB fusion proteins was grown overnight at 37°C in Luria broth (46) containing 100 μg of carbenicillin per ml. These were subcultured in the same medium to an optical density at 600 nm ($OD_{600}$) of 0.1 and were grown to an $OD_{600}$ of ~0.8, at which point the cultures were induced with various concentrations of IPTG (0.05 to 1.2 mM) for 45 min. The $OD_{600}$s of the cultures were determined, and the cells were centrifuged (2,000 × *g* for 5 min) and resuspended to $10^9$ cells per ml in phosphate-buffered saline (PBS; pH 7.4) containing 0.04% glucose and 10 mM $MgSO_4$. An aliquot of cells was used in the antigen-processing assay for either MHC-I- or MHC-II- restricted antigen presentation (see below), and another aliquot was used to quantitate the HEL or OVA epitope by enzyme-linked immunosorbent assay (ELISA) as described below. Growth of *E. coli* HB101 constitutively expressing the Crl-HEL or Crl-OVA fusion protein was on Luria agar plates supplemented with 100 μg of carbenicillin per ml. Cells were removed from the plates by washing with PBS (pH 7.4) containing 0.04% glucose and 10 mM $MgSO_4$.

**Quantitation of the HEL or OVA epitope in induced cultures.** An aliquot of cells at $10^9$ cells per ml was centrifuged, resuspended in 10 mM Tris (pH 7.6), and lysed by sonication on ice four times for 20 s each (at setting 45 on a Biosonic Sonicator; Bronwell Scientific, Rochester, N.Y.). Unbroken cells were removed by centrifugation, and the total protein contents of the cleared sonicates were determined by the bicinchoninic acid protein determination system (Sigma, St. Louis, Mo.). All samples were standardized to the same total protein concentration and were serially diluted in PBS (pH 7.4), and 100-μl aliquots were seeded in triplicate in 96-well microtiter plates (Nunc Immunosorp). ELISAs were carried out as previously described (43). The primary antiserum used to detect HEL(45–62) was raised in rabbits against synthetic HEL(34–61) peptide conjugated to ovalbumin. The primary antiserum used to detect OVA(256–265) was raised

against native ovalbumin in C57BL/6 mice. Prior to use in ELISA, each primary antiserum was first adsorbed with an acetone powder (25) made from RAM191/pUHE21-2. Secondary antiserum (1:1,000) was either anti-rabbit immunoglobulin G or anti-mouse immunoglobulin G peroxidase conjugate (Sigma). The quantities of the HEL and the OVA epitopes in the induced samples were determined from a standard curve prepared by diluting pure native chicken egg white lysozyme [for HEL(45–62) determinations] or chicken egg ovalbumin [for OVA(256–265) determinations] in RAM191/pUHE21-2-cleared sonicate at the same total protein concentrations as those for all other sonicates. The background reactivity of RAM191/pUHE21-2 was subtracted from all test samples prior to quantitation of epitope in the lysates.

**SDS-PAGE and Western blot (immunoblot) analysis.** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 30) and Western blot analysis (25) were performed as described elsewhere.

**Maltoporin function determination, bacteriophage assays, and whole-cell ELISAs.** Maltoporin function of *E. coli* RAM191 containing pUHE21-2, pLamBHEL153, pLamBHEL183, pLamBOVA153, or pLamBOVA183, as well as of MC4100 (the wild-type *lamB* strain from which RAM191 was derived), was determined as previously described (37). Briefly, with M63 minimal medium containing 50 μg of carbenicillin (Sigma) per ml, 0.05 mM IPTG, and a single maltodextrin (1 mM) as the sole carbon source, cultures were grown at 37°C with shaking. Periodically, aliquots were removed, and the $OD_{600}$ was determined. The carbon sources tested were glucose, maltose, maltotriose, maltotetraose, maltopentaose, and maltohexaose (Sigma).

Bacteriophage assays were carried out on *E. coli* RAM191 containing pUHE21-2, pLamBHEL153, pLamBHEL183, pLamBOVA153, or pLamBOVA183, as well as on MC4100. Cultures were grown overnight in Luria broth supplemented with 10 mM $MgSO_4$ and 0.2% maltose. An aliquot of these cultures was mixed with H soft agar containing either 0, 0.01, 0.1, or 1 mM IPTG, and the mixtures were plated on H plates (36). Sensitivity to wild-type bacteriophage lambda was assessed qualitatively by determining plaque formation within 12 h of plating the samples.

Whole-cell ELISAs were carried out as previously described (11). Briefly, bacterial strains were grown and induced for fusion protein expression as described above, and $3 \times 10^6$ bacteria per well were coated in 96-well microtiter plates (Nunc Immunosorp). After incubation at room temperature for 2.5 h, the wells were washed with PBS (pH 7.4) and were blocked overnight at 4°C with 3% bovine serum albumin in PBS (fraction V; Sigma). The wells were incubated with anti-HEL serum and, after being washed with PBS, were incubated with anti-rabbit immunoglobulin G peroxidase conjugate secondary antiserum. Reactivity with the HEL antiserum was quantitated by reading the $OD_{450}$ after incubation of the plates with the chromogenic substrate 0.04% *O*-phenylenediamine dihydrochloride (diluted in 0.1 M sodium phosphate, 0.05 M citric acid, and 0.04% hydrogen peroxide [pH 5.0]).

**Antigen-processing assay.** Phagocytic processing of microbes expressing a HEL or an OVA fusion protein for MHC-II- or MHC-I-restricted antigen presentation, respectively, was quantitated as previously described (42, 43). Briefly, peritoneal macrophages were elicited in either CBA/J ($H\text{-}2^k$) or C57BL/6 ($H\text{-}2^b$) mice (Jackson Laboratory, Bar Harbor, Maine) by intraperitoneal injection with *Listeria monocytogenes* (24) and were collected 7 to 14 days later. *E. coli* induced for fusion protein expression was added to $2 \times 10^5$ adherent macrophages in antibiotic-free medium, and after gentle centrifugation ($1,000 \times g$ for 5 min), the cultures were incubated at 37°C for 2 h. After washing, the cells were fixed in 1% paraformaldehyde for 15 min and were extensively washed. A total of $10^5$ 3A9 T hybridoma cells (39) or CD8OVA T hybridoma cells (42) specific for the HEL(52–61) or the OVA(257–264) epitopes, respectively, was then added for a 24-h incubation. These hybridoma cells secrete interleukin-2 upon specific recognition of the appropriate MHC-peptide complex. Interleukin-2 production by the T hybridoma cells was quantitated with interleukin-2-dependent CTLL cells.

## RESULTS

**Characterization of HEL and OVA fusion proteins.** One aim of our study was to examine the effect of localization of an epitope within a bacterium on phagocytic antigen processing. Previous reports describe the use of the *E. coli* outer membrane protein LamB as a protein suited for expression of foreign epitopes in specific compartments of the microbe (8, 10, 11, 38). For example, insertion of foreign epitopes after residue 153 of LamB exposes that epitope on the surface of *E. coli* and insertion of an epitope after amino acid 183 results in epitopes which face the periplasmic space (8, 10, 11, 38). Thus, the HEL(45–62) and the OVA(256–265) epitopes were inserted into LamB after amino acids 153 and 183 to create fusion proteins expressing each epitope on the bacterial surface or facing the periplasm, respectively (Fig. 1; Table 1).

To confirm the function and localization of the LamB fusion proteins, three characteristics were examined. First, growth of *E. coli* RAM191 expressing a LamB fusion protein was examined in minimal medium containing a single high-molecular-weight maltodextrin as the sole carbon source, conditions in which LamB must exhibit maltoporin function for growth of the bacteria (37). Under these conditions, the doubling times of RAM191/pLamBHEL153, RAM191/pLamBHEL183, RAM191/pLamBOVA153, RAM191/pLamBOVA183, and MC4100 (the wild-type *lamB* parent strain) were all <4 h on maltotetraose, maltopentaose, or maltohexaose. The vector control (RAM191/pUHE21-2) had a doubling time of >43.5 h under these conditions. This demonstrates the outer membrane localization of the fusion proteins and their competence for maltoporin transport. In addition, since the LamB protein is the receptor for bacteriophage lambda (13), the *lamB* deletion strain *E. coli* RAM191 expressing a plasmid-encoded LamB fusion protein was tested for its sensitivity to bacteriophage lambda in a plate plaque assay with lambda with a wild-type host range. Whereas RAM191 expressing LamBHEL153 or LamBOVA153 was not sensitive to this bacteriophage, RAM191 expressing LamBHEL183 or LamBOVA183 was sensitive to this phage. This is consistent with previous reports in which LamB fused with an epitope after position 183 was sensitive to bacteriophage lambda, whereas LamB fused with an epitope after position 153 resulted in LamB fusions with altered lambda sensitivities (10, 11), presumably because of disruption of the surface epitope required for lambda binding. Finally, whole-cell ELISA analysis of RAM191/pLamBHEL153 revealed that the ELISA reactivity (using anti-HEL serum as the primary antiserum) was 15 times above the reactivity of RAM191/pUHE21-2, indicating that the HEL epitope in this construct is exposed on the bacterial surface. The reactivity of RAM191/pLamBHEL183 was essentially the same as the

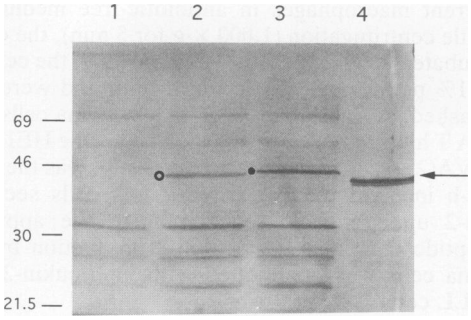


FIG. 2. Western blot of LamBHEL and MalEHEL fusion proteins. Cleared bacterial lysates from induced cultures of *E. coli* containing the indicated plasmids were separated by SDS-10% PAGE and were processed for Western blotting to detect the HEL epitope. The migration of molecular mass markers in kilodaltons is indicated. Lanes: 1, *E. coli* RAM191/pUHE21-2 (vector control); 2, *E. coli* RAM191/pLamBHEL153; 3, *E. coli* RAM191/pLamBHEL183; 4, *E. coli* RAM191/pMalEHEL. The fusion proteins are indicated with either an arrow (MalEHEL), a closed circle (LamBHEL183), or an open circle (LamBHEL153).

reactivity of the vector control (RAM191/pUHE21-2), indicating that the HEL epitope in LamBHEL183 is not surface exposed. Similar results were obtained for RAM191 expressing the OVA fusion proteins (42). Taken together, these data support the previously proposed model for the topology of LamB such that epitopes fused after LamB residue 153 are on the exterior surface of *E. coli*, and fusions after LamB residue 183 face the periplasmic space (11). Furthermore, these fusions do not severely alter the structure or the function of LamB.

HEL and OVA fusion proteins that remain in the cytoplasm of *E. coli* were initially generated by PCR to delete the *lamB* signal sequence in pLamBHEL153 or pLamBOVA153 to prevent secretion of these LamB fusions into the periplasmic space (18). ELISA analysis showed that after induction for fusion protein expression, only a minor fraction of the epitope was present in the soluble fraction of bacterial sonicates; Western blot analysis on boiled cell pellets obtained after centrifugation of sonicated samples confirmed that the majority of induced protein remained in the cell pellets (data not shown). This suggested that the LamB fusion proteins lacking the signal sequence accumulated in an insoluble form in the bacterial cytoplasm. Therefore, to make soluble cytoplasmic HEL and OVA fusion proteins, an alternate strategy was used to fuse a portion of LamB that already contained either the HEL(45–62) or the OVA(256–265) epitope to the carboxy terminus of the mature *E. coli* MalE protein (Fig. 1). Although MalE is a periplasmic component of the maltose transport system of *E. coli* (17), deletion of its signal sequence localizes the mature MalE protein in the bacterial cytoplasm in a soluble form (4) (Fig. 2). Fusion proteins with molecular masses of approximately 46,000 and 41,000 Da, the predicted sizes for the LamBHEL and MalEHEL fusion proteins, respectively, were present in Western blot analysis of cleared sonicates of induced bacterial cultures (Fig. 2). Identical results were obtained for the LamBOVA and MalEOVA fusion proteins (data not shown).

**Expression of HEL and OVA fusion proteins.** Late-logarithmic-phase cultures of *E. coli* harboring the LamB fusion protein plasmids were induced for fusion protein expression by the addition of IPTG. With this inducible system, various levels of expression could be obtained which allowed the effect of increased epitope expression on antigen processing to be examined. In addition, by induction of the fusion proteins localized in the various bacterial compartments to the same level of expression, the effect of compartmentalization of an epitope within the bacterium on antigen processing could be examined.

Quantitative ELISA with either anti-HEL or anti-OVA serum allowed the levels of expression of all fusion proteins to be monitored in cleared bacterial sonicates. Increased amounts of IPTG added to a bacterial culture resulted in a concomitant increase in fusion protein detected in cleared bacterial sonicates (see relative amounts in Fig. 3 to 7). With a given level of inducer, the fusion proteins were expressed at approximately the same level. Induction of the membrane-competent fusion proteins (LamBHEL153, LamBHEL183, LamBOVA153, and LamBOVA183) resulted in a somewhat reduced bacterial growth rate that corresponded with increased IPTG addition (data not shown). Such growth alteration is predicted according to previous reports in which overexpression of LamB becomes toxic to cells when expressed at very high levels (11). Under the induction conditions described, a number of expression levels was achieved without reaching toxicity for the cells. The constitutively expressed fusion proteins Crl-HEL (43) and Crl-OVA (42) were present in amounts approximately 10- to 100-fold greater than those of the LamB or MalE proteins induced as described here. This is a likely result on the basis of the high copy number of the pUC18 vector of the Crl fusion proteins and the constitutive expression from the native *crl* promoter (42, 43).

**Processing of *E. coli* expressing HEL fusion proteins.** Macrophage processing of the HEL epitope present in the outer membrane of *E. coli* exposed either at the cell surface (LamBHEL153) or in the periplasmic space (LamBHEL183) resulted in significant presentation to HEL(52–61)-specific T hybridoma cells (Fig. 3 and 4). Likewise, soluble cytoplasmic HEL epitope in the MalEHEL fusion protein was processed for presentation to epitope-specific T hybridoma cells (Fig. 5).

The effect of various amounts of HEL epitope on processing for MHC-II presentation was examined by inducing *E. coli* to express a single HEL fusion protein at various levels. Analysis within the linear range of the antigen-processing assay revealed that increased abundance of the HEL epitope, regardless of the bacterial compartment within which the epitope was contained, resulted in increased MHC-II-associated presentation (Fig. 3, surface HEL; Fig. 4, periplasmic HEL; Fig. 5, cytoplasmic HEL). To ensure that this presentation resulted from phagocytic processing of intact microbes rather than proteins released from lysed bacteria, inhibition studies with cytochalasin D were performed. Treatment of macrophages with cytochalasin D, which interferes with actin polymerization and consequently inhibits the phagocytic capability of macrophages, completely abolished presentation of surface-exposed HEL epitope (Fig. 3). Identical inhibition results were obtained when the HEL epitope was expressed in other bacterial compartments (data not shown).

To examine the effect of compartmentalization of the HEL epitope in bacteria on processing for MHC-II presentation, *E. coli* strains expressing the same epitope in different bacterial compartments (surface, periplasm, or cytoplasm) were induced to produce similar quantities of epitope. Under these conditions, minor differences in processing efficiency were detected, and processing of HEL(45–62) exposed in the periplasmic space by LamBHEL183 was the most efficient

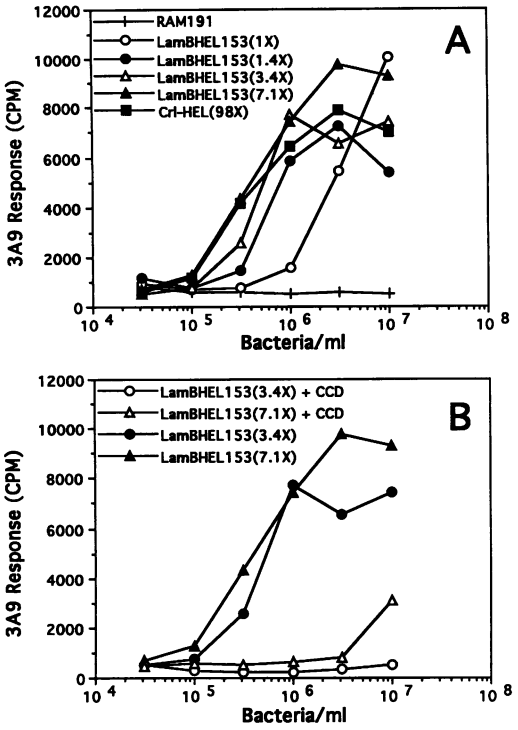


FIG. 3. Processing of *E. coli* cells expressing surface-localized HEL epitope for presentation via MHC-II. (A) Response of 3A9 T hybridoma cells to *E. coli* RAM191/pLamBHEL153 induced to achieve various expression levels of LamBHEL153 fusion protein. The relative abundance of HEL epitope expressed by bacteria induced in this experiment as quantitated by ELISA is indicated in parentheses. (B) Cytochalasin D (CCD) inhibits processing of surface-localized HEL epitope. *E. coli* RAM191/pLamBHEL153 (cells induced to relative expression levels of 3.4 and 7.1 times) were incubated with macrophages with (+) or without addition of cytochalasin D at 10 μg/ml beginning 10 min prior to the addition of bacteria. The data are from the experiment shown in panel A.

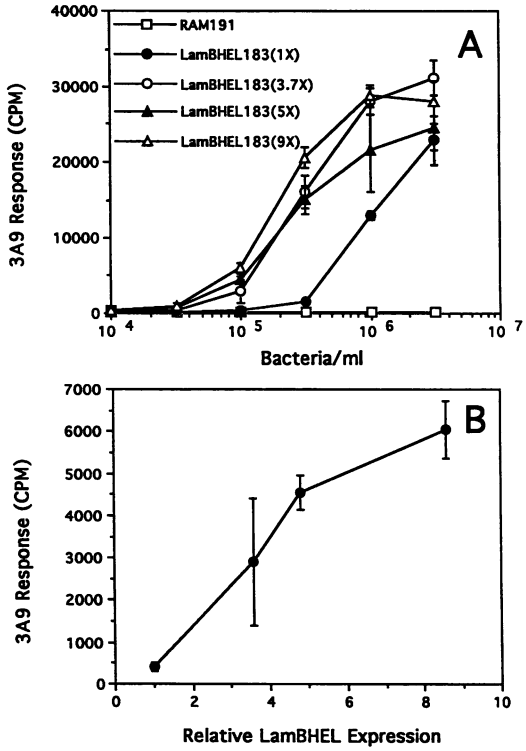


FIG. 4. Processing of *E. coli* cells expressing HEL epitope facing the periplasmic space for presentation via MHC-II. (A) Response of 3A9 T hybridoma cells to *E. coli* RAM191/pLamBHEL183 induced to achieve various levels of expression of LamBHEL183 fusion protein. The relative levels of HEL epitope expressed by bacteria induced in this experiment as quantitated by ELISA are indicated in parentheses. (B) Response to $10^5$ *E. coli* RAM191/pLamBHEL183 expressed as a function of the relative LamBHEL183 expression level. Error bars represent ± 1 standard deviation for triplicate samples.

compared with the surface exposed or cytoplasmic epitopes (Fig. 5). Processing of HEL(45–62) from the cytoplasmically expressed MalEHEL fusion protein was the least efficient, whereas processing efficiency of the surface localized epitope in LamBHEL153 fell between the periplasmic and the cytoplasmic fusion proteins (Fig. 5). These minor differences in processing efficiency, which may result from differences either in local protein context or bacterial compartmentalization of the epitope, can be overcome by increasing the amount of epitope per bacterium two to four times (Fig. 5).

**Processing of *E. coli* expressing OVA fusion proteins.** We previously demonstrated a novel phagocytic pathway whereby epitopes expressed in bacteria are processed and presented by MHC-I molecules (42). Here, we examined the effect of epitope abundance and compartmentalization within the microbe on this processing pathway. Within the linear portion of the assay system, increased amounts of OVA fusion protein resulted in increased antigen presentation to CD8OVA T hybridoma cells regardless of the compartmentalization of the epitope within *E. coli* (Fig. 6 and 7). However, with similar levels of OVA(256–265) expression, the epitope facing the periplasmic space in LamBOVA183 was processed more efficiently than the surface-exposed epitope in LamBHEL153 (Fig. 7A). In addition, the cytoplasmic fusion protein (MalEOVA) was processed more efficiently than the fusion protein containing surface-exposed epitope (Fig. 7B). Thus, the order of efficiency for MHC-I presentation of fusion proteins containing OVA(256–265) appears to be the same as that observed for MHC-II presentation of fusions containing HEL(45–62).

## DISCUSSION

Construction and characterization of recombinant bacterial strains expressing foreign epitopes from virulence-associated proteins of many microbial pathogens have been pursued as a means of producing safe and efficacious vaccines. Many recombinant strains have been specifically designed to express surface-exposed epitopes with the idea that such an epitope would be readily accessible to the immune system. However, it is unclear whether any immunologic advantage is provided by expressing a foreign epitope at the surface of a recombinant bacterial strain compared with expression in a compartment within the bacterium. In addition, expressing epitopes as fusions to membrane proteins may result in toxicity to the host bacterium and compromise its growth. Thus, even though some expression systems such as LamB offer the option of expressing foreign epitopes on the bacterial surface, the toxic

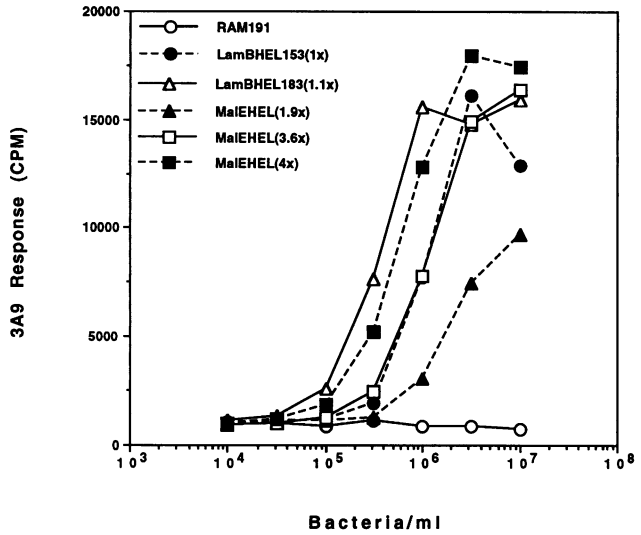


FIG. 5. Processing of *E. coli* cells expressing the HEL epitope in various bacterial compartments for presentation via MHC-II. The 3A9 response to *E. coli* RAM191 expressing fusion proteins with the HEL epitope exposed either on the surface (LamBHEL153), facing the periplasmic space (LamBHEL183), or in the cytoplasm (MalE-HEL) is shown. The relative levels of HEL epitope expressed by bacteria induced in this experiment as quantitated by ELISA are indicated in parentheses.

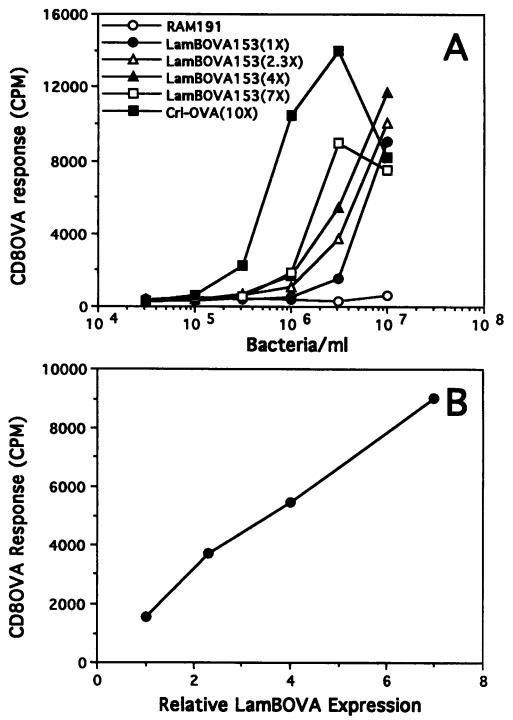


FIG. 6. Processing of *E. coli* cells expressing surface-localized OVA epitope for presentation via MHC-I. (A) Response of CD8OVA T hybridoma cells to *E. coli* RAM191/pLamBOVA153 induced to achieve various levels of LamBOVA153 fusion protein. The relative levels of OVA epitope expressed by bacteria induced in this experiment as quantitated by ELISA are indicated in parentheses. (B) The CD8OVA response to $3 \times 10^6$ *E. coli* RAM191/pLamB OVA153 cells expressed as a function of the relative LamBOVA153 expression level.

effect of protein overexpression limits the usefulness of such systems for high-level production of an epitope within a microbe for potential vaccine use. In addition, cloning foreign epitopes into surface appendages that protrude out from bacteria such as pili (26, 48) or flagella (29, 40) may result in hybrid proteins that are incapable of polymerizing into a functional organelle on the exterior of the bacteria or that have altered structures when the epitope is inserted (26, 48, 49). These factors may compromise the usefulness of surface-exposed expression systems as host proteins for foreign epitopes.

The level of expression of a given epitope, regardless of the bacterial compartmentalization of the epitope, strongly correlated with the efficiency of processing for both MHC-I and MHC-II presentation to T cells. Interestingly, expression of either the HEL or the OVA epitope in the cytoplasm of *E. coli* cells as an insoluble fusion protein (in LamB lacking the signal sequence) resulted in very inefficient phagocytic processing (data not shown). The decrease in processing of these epitopes from *E. coli* correlated with a reduction in soluble epitope present in cleared bacterial sonicates detected by ELISA, and Western blot analysis of boiled whole cells revealed that the majority of the epitope expressed in these fusion proteins remained in an insoluble form in the cell pellet, probably in inclusion bodies. This suggests that epitope present as an insoluble cytoplasmic fusion protein is not efficiently processed for MHC-I or MHC-II presentation to T cells (5, 20). It may be that the appropriate peptides are not efficiently generated in the correct phagolysosomal compartment in this case.

The in vitro antigen-processing system utilized here allowed a systematic study of the effect of epitope compartmentalization within bacteria on epitope processing. Our data show that the epitopes that are expressed in LamB such that they face the periplasmic space were the most efficiently processed and that expression of an epitope within different bacterial compartments had at most a minor effect on the efficiency of processing for both MHC-I and MHC-II presentation to T hybridoma cells (Fig. 5 and 7). The minor differences in the efficiency of epitope processing may be due to the local protein context of the epitope as well as to bacterial compartmentalization. The surface and cytoplasmic fusion proteins provide the most stringent basis for comparison to assess the effect of epitope compartmentalization on processing efficiency, since the epitope localized in these two fusion proteins is identical in the 11 and 12 amino acids flanking the amino and carboxyl ends of the epitope, respectively (Fig. 1). Comparison of epitope processing from these fusion proteins suggests that surface-exposed epitope is slightly more efficiently processed for presentation to T cells than cytoplasmic epitope, but this difference can be overcome by expressing as little as two to five times more epitope in the cytoplasm (Fig. 5 and 7). Thus, the minor differences in processing efficiency among the various constructs that we observed may relate to the protein context of the epitope. In particular, localization to the surface did not provide the most efficient epitope processing.

Thus, it appears that there is no distinct advantage in any particular bacterial compartment for epitope processing. Minor differences in processing efficiency due to either bacterial compartmentalization or the local protein context of the epitope can be readily overcome by simply increasing the relative amount of epitope per bacterium. Consistent

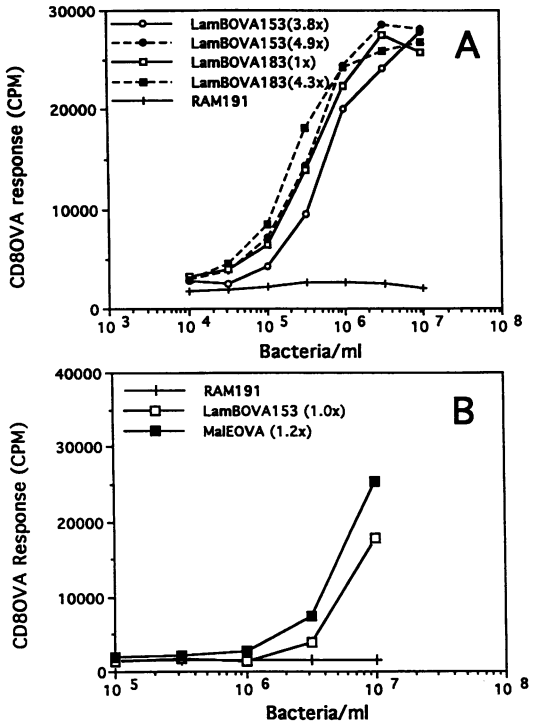


FIG. 7. Processing of *E. coli* cells expressing the OVA epitope in various bacterial compartments for presentation via MHC-I. (A) Response of CD8OVA T hybridoma cells to *E. coli* RAM191 expressing fusion proteins with the OVA epitope exposed either on the surface (LamBOVA153) or facing the periplasmic space (LamB OVA183). (B) The CD8OVA response to *E. coli* RAM191 expressing either surface (LamBOVA153) or cytoplasmic (MalEOVA) OVA epitope. The relative levels of OVA epitope expressed by bacteria induced in this experiment as quantitated by ELISA are indicated in parentheses.

with this notion is our observation that *E. coli* expressing the constitutively expressed, highly abundant cytoplasmic epitopes in the Crl-HEL and Crl-OVA fusion proteins gave the most efficient processing for both the MHC-I and MHC-II presentation pathways (Fig. 3 and 6). These fusion proteins, which have between 10 and 100 times more epitope than the LamB fusion proteins, illustrate the usefulness for effective T-cell stimulation of a constitutive expression system that produces abundant cytoplasmic epitope.

These data also have implications for the mechanisms of antigen processing within macrophages. Both intracellular and extracellular epitopes are efficiently processed, including both soluble and membrane proteins. Thus, the antigen processing compartment must contain extensive degradative capacity, consistent with phagolysosomal properties. This is in agreement with our prior data implicating macrophage phagolysosomes and lysosomes in antigen processing for MHC-II presentation (15, 21, 22, 43). MHC-II molecules have recently been shown to be present in lysosomes that fuse with phagosomes containing bacteria (23), and thus it is likely that bacterial peptides produced by phagolysosomal catabolism bind directly to MHC-II molecules and are then expressed on the cell surface.

The mechanisms for phagocytic processing of bacterial epitopes for MHC-I presentation remain less clear, since this pathway has only recently been elucidated (42). However, phagolysosomal catabolism appears necessary for this pathway as well. Peptides may then bind to MHC-I molecules within an intracellular compartment (perhaps the phagolysosome itself), or they may be released to bind to surface MHC-I molecules (42).

In summary, the data presented here indicate that phagocytic processing of epitopes present in intact bacteria occurs regardless of bacterial compartmentalization, with epitope abundance being the most critical factor. The design of recombinant bacterial vaccine strains to elicit T-cell responses should therefore utilize systems that allow abundant epitope expression in any bacterial compartment. Expression of an epitope as a fusion protein within a bacterium may allow abundant production without toxicity to the bacterium itself, yielding very high levels of epitope expression to maximize processing efficiency for presentation to T cells.

## ACKNOWLEDGMENTS

We gratefully acknowledge Igor Brikun for helpful discussions and S. A. Benson for *E. coli* RAM191.

This work was supported by W. M. Keck postdoctoral fellowships awarded to M.J.W. and J.D.P. C.V.H. was supported by an American Cancer Society Junior Faculty Research award and a Pfizer Scholar award.

## REFERENCES

1. **Aggarwal, A., S. Kumar, R. Jaffe, D. Hone, M. Gross, and J. Sadoff.** 1990. Oral *Salmonella*: malaria circumsporozoite recombinants induce specific $CD8^+$ cytotoxic T cells. J. Exp. Med. **172:**1083–1090.
2. **Agterberg, M., H. Adriaanse, H. Lankhof, R. Meloen, and J. Tommassen.** 1990. Outer membrane PhoE protein of *Escherichia coli* as a carrier for foreign antigenic determinants: immunogenicity of epitopes of foot-and-mouth disease virus. Vaccine **8:**85–91.
3. **Amann, E., B. Ochs, and K. J. Abel.** 1988. Tightly regulated tac promoter vectors useful for the expression of unfused and fused proteins in *Escherichia coli*. Gene **69:**301–315.
4. **Bankaitis, V. A., B. A. Rasmussen, and P. J. Bassford, Jr.** 1984. Intragenic suppressor mutations that restore export of maltose binding protein with a truncated signal peptide. Cell **37:**243–252.
5. **Brett, S. J., J. Rhodes, F. Y. Liew, and J. P. Tite.** 1993. Comparison of antigenic presentation of influenza A nucleoprotein expressed in attenuated $AroA^-$ *Salmonella typhimurium* with that of live virus. J. Immunol. **150:**2869–2884.
6. **Calos, M. P.** 1978. DNA sequence for a low-level promoter of the *lac* repressor gene and an "up" promoter mutation. Nature (London) **274:**762–765.
7. **Casadaban, M. J.** 1976. Transposition and fusion of the *lac* genes to selected promoters in *Escherichia coli* using bacteriophage lambda and Mu. J. Mol. Biol. **104:**541–555.
8. **Charbit, A., J. C. Boulain, A. Ryter, and M. Hofnung.** 1986. Probing the topology of a bacterial membrane protein by genetic insertion of a foreign epitope; expression at the cell surface. EMBO J. **5:**3029–3037.
9. **Charbit, A., A. Molla, J. Ronco, J.-M. Clément, V. Favier, E. M. Bahraoui, L. Montagnier, A. Leguern, and M. Hofnung.** 1990. Immunogenicity and antigenicity of conserved peptides from the envelope of HIV-1 expressed at the surface of recombinant bacteria. AIDS **4:**545–551.
10. **Charbit, A., A. Molla, W. Saurin, and M. Hofnung.** 1988. Versatility of a vector for expressing foreign polypeptides at the surface of gram-negative bacteria. Gene **70:**181–189.
11. **Charbit, A., J. Ronco, V. Michel, C. Werts, and M. Hofnung.** 1991. Permissive sites and topology of an outer membrane protein with a reporter epitope. J. Bacteriol. **173:**262–275.
12. **Charbit, A., E. Sobczak, M.-L. Michel, A. Molla, P. Tiollais, and M. Hofnung.** 1987. Presentation of two epitopes of the preS2 region of hepatitis B virus on live recombinant bacteria. J. Immunol. **139:**1658–1664.
13. **Clément, J. M., and M. Hofnung.** 1981. Gene sequence of the

lambda receptor, an outer membrane protein of *E. coli* K12. Cell **27**:507–514.
14. **Clements, J. D., F. L. Lyon, K. L. Lowe, A. L. Farrand, and S. El-Morshidy.** 1986. Oral immunization of mice with attenuated *Salmonella enteritidis* containing a recombinant plasmid which codes for production of the B subunit of heat-labile *Escherichia coli* enterotoxin. Infect. Immun. **53**:685–692.
15. **Collins, D. S., E. R. Unanue, and C. V. Harding.** 1991. Reduction of disulfide bonds within lysosomes is a key step in antigen processing. J. Immunol. **147**:4054–4059.
16. **Dougan, G., R. Sellwood, D. Maskell, K. Sweeney, F. Y. Liew, J. Beesley, and C. Hormaeche.** 1986. In vivo properties of a cloned K88 adherence antigen determinant. Infect. Immun. **52**:344–347.
17. **Duplay, P., H. Bedouelle, A. Fowler, I. Zabin, W. Saurin, and M. Hofnung.** 1984. Sequences of the *MalE* gene and of its product, the maltose-binding protein of *Escherichia coli* K12. J. Biol. Chem. **259**:10606–10613.
18. **Emr, S. D., J. Hedgpeth, J.-M. Clément, T. J. Silhavy, and M. Hofnung.** 1980. Sequence analysis of mutations that prevent export of lambda receptor, an *Escherichia coli* outer membrane protein. Nature (London) **285**:82–85.
19. **Falk, K., O. Rötzschke, S. Stevanović, G. Jung, and H.-G. Rammensee.** 1991. Allele-specific motifs revealed by sequencing of self-peptides eluted from MHC molecules. Nature (London) **351**:290–296.
20. **Gao, X.-M., J. P. Tite, M. Lipscombe, S. Rowland-Jones, D. J. P. Ferguson, and A. J. McMichael.** 1992. Recombinant *Salmonella typhimurium* strains that invade nonphagocytic cells are resistant to recognition by antigen-specific cytotoxic T lymphocytes. Infect. Immun. **60**:3780–3789.
21. **Harding, C. V., D. S. Collins, O. Kanagawa, and E. R. Unanue.** 1991. Liposome-encapsulated antigens engender lysosomal processing for class II MHC presentation and cytosolic processing for class I presentation. J. Immunol. **147**:2860–2863.
22. **Harding, C. V., D. S. Collins, J. W. Slot, H. J. Geuze, and E. R. Unanue.** 1991. Liposome-encapsulated antigens are processed in lysosomes, recycled, and presented to T cells. Cell **64**:393–401.
23. **Harding, C. V., and H. J. Gueze.** 1992. Class II MHC molecules are present in macrophage lysosomes and phagolysosomes that function in the phagocytic processing of Listeria monocytogenes for presentation to T cells. J. Cell Biol. **119**:531–542.
24. **Harding, C. V., R. W. Roof, and E. R. Unanue.** 1989. Turnover of Ia-peptide complexes is facilitated in viable antigen-presenting cells: biosynthetic turnover of Ia vs. peptide exchange. Proc. Natl. Acad. Sci. USA **86**:4230–4234.
25. **Harlow, E., and D. Lane.** 1988. Antibodies: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
26. **Hedegaard, L., and P. Klemm.** 1989. Type 1 fimbriae of *Escherichia coli* as carriers of heterologous antigenic sequences. Gene **85**:115–124.
27. **Jacob, C. O., M. Leitner, A. Zamir, D. Salomon, and R. Arnon.** 1985. Priming immunization against cholera toxin and *E. coli* heat-labile toxin by a cholera toxin short peptide-β-galactosidase hybrid synthesized in *E. coli*. EMBO J. **4**:3339–3343.
28. **Jung, A., A. E. Sippel, M. Grez, and G. Schütz.** 1980. Exons encode functional and structural units of chicken lysozyme. Proc. Natl. Acad. Sci. USA **77**:5759–5763.
29. **Kuwajima, G., J.-I. Asaka, T. Fujiwara, T. Fujiwara, K. Nakano, and E. Kondoh.** Presentation of an antigenic determinant from hen egg-white lysozyme on the flagellar filament of *Escherichia coli*. Bio/Technology **6**:1080–1083.
30. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
31. **Lanzer, M., and H. Bujard.** 1988. Promoters largely determine the efficiency of repressor action. Proc. Natl. Acad. Sci. USA **85**:8973–8977.
32. **Leclerc, C., A. Charbit, A. Molla, and M. Hofnung.** 1989. Antibody response to a foreign epitope expressed at the surface of recombinant bacteria: importance of the route of immunization. Vaccine **7**:242–248.
33. **Leclerc, C., P. Martineau, S. van der Werf, E. Deriaud, P. Duplay, and M. Hofnung.** 1990. Induction of virus-neutralizing antibodies by bacteria expressing the C3 poliovirus epitope in the periplasm. The route of immunization influences the isotypic distribution and the biologic activity of the antipoliovirus antibodies. J. Immunol. **144**:3174–3182.
34. **Maskell, D. J., K. J. Sweeney, D. O'Callaghan, C. E. Hormaeche, F. Y. Liew, and G. Dougan.** 1987. *Salmonella typhimurium aroA* mutants as carriers of the *Escherichia coli* heat-labile enterotoxin B subunit to the murine secretory and systemic immune systems. Microb. Pathog. **2**:211–221.
35. **McReynolds, L., B. W. O'Malley, A. D. Nisbet, J. E. Fothergill, D. Givol, S. Fields, M. Robertson, and G. G. Brownlee.** 1978. Sequence of chicken ovalbumin mRNA. Nature (London) **273**:723–728.
36. **Miller, J. H.** 1992. A short course in bacterial genetics. A laboratory manual and handbook for *Escherichia coli* and related bacteria. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
37. **Misra, R., and S. A. Benson.** 1988. Isolation and characterization of OmpC porin mutants with altered pore properties. J. Bacteriol. **170**:528–533.
38. **Molla, A., A. Charbit, A. Le Guern, A. Ryter, and M. Hofnung.** 1989. Antibodies against synthetic peptides and the topology of LamB, an outer membrane protein from *Escherichia coli* K12. Biochemistry **28**:8234–8241.
39. **Nelson, C. A., R. W. Roof, D. W. McCourt, and E. R. Unanue.** 1992. Identification of the naturally processed form of hen egg white lysozyme bound to the murine major histocompatibility complex class II molecule I-$A^k$. Proc. Natl. Acad. Sci. USA **89**:7380–7383.
40. **Newton, S. M. C., C. O. Jacob, and B. A. D. Stocker.** 1989. Immune response to cholera toxin epitope inserted in *Salmonella* flagellin. Science **244**:70–72.
41. **O'Callaghan, D., A. Charbit, P. Martineau, C. Leclerc, S. van der Werf, C. Nauciel, and M. Hofnung.** 1990. Immunogenicity of foreign peptide epitopes expressed in bacterial envelope proteins. Res. Microbiol. **141**:963–969.
42. **Pfeifer, J. D., M. J. Wick, R. L. Roberts, K. Findlay, S. J. Normark, and C. V. Harding.** 1993. Phagocytic processing of bacterial antigens for class I MHC presentation to T cells. Nature (London) **361**:359–362.
43. **Pfeifer, J. D., M. J. Wick, D. G. Russell, S. J. Normark, and C. V. Harding.** 1992. Recombinant *Escherichia coli* express a defined, cytoplasmic epitope that is efficiently processed in macrophage phagolysosomes for class II MHC presentation to T lymphocytes. J. Immunol. **149**:2576–2584.
44. **Poirier, T. P., M. A. Kehoe, and E. H. Beachey.** 1988. Protective immunity evoked by oral administration of attenuated *aroA Salmonella typhimurium* expressing cloned streptococcal M protein. J. Exp. Med. **168**:25–32.
45. **Rickenberg, H. V., and G. Lester.** 1955. The preferential systhesis of β-galactosidase in *Escherichia coli*. J. Gen. Microbiol. **13**:279–284.
46. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
47. **van der Werf, S., A. Charbit, C. Leclerc, V. Mimic, J. Ronco, M. Girard, and M. Hofnung.** 1990. Critical role of neighbouring sequences on the immunogenicity of the C3 poliovirus neutralization epitope expressed at the surface of recombinant bacteria. Vaccine **8**:269–277.
48. **van Die, I., M. Wauben, I. van Megen, H. Bergmans, N. Riegman, W. Hoekstra, P. Pouwels, and B. Enger-Valk.** 1988. Genetic manipulation of major P-fimbrial subunits and consequences for formation of fimbriae. J. Bacteriol. **170**:5870–5876.
49. **Wick, M. J., and S. J. Normark.** Unpublished data.