preserved and not lost through decreased viability correlated with high-level expression and overgrowth by nonexpressing or nonfunction cells. Finally, HIV reverse transcriptase has been coupled to the Lpp-OmpA dibrid and the tripartite fusion localized to the OM of an attenuated (Aro⁻) Salmonella strain. Following oral administration in mice, mucosal IgA and T-cell responses were elicited. The available evidence, therefore, indicates that the Lpp-OmpA surface display vehicle may function well in several categories of biotechnological application.

Final Comments

Many molecular details of the action of the Lpp-OmpA chimeric vehicle remain to be elucidated, including whether the Lpp export pathway is used (it differs from that of nonlipoproteins in having a different signal peptidase and in requiring a specific chaperone to reach the OM), whether fusion proteins exist as monomers or multimers in the OM, if concurrent LPS synthesis is necessary for OM insertion of the tribrid, which specific proteins involved in folding nascent polypeptides are required and if the same ensemble of these proteins is used with every passenger protein, and to what extent the various tribrids trigger the several bacterial stress response systems. Answers to these questions may provide additional ideas for extending and optimizing the Lpp-OmpA-based surface display procedure. Whether host/Lpp-OmpA-encoding plasmid systems capable of displaying any soluble protein can be developed, regardless of passenger size, topology, normal location, or possibility of quaternary structure, remains to be seen.

M. S. Burnett, M. Hofmann, and G. B. Kitto, submitted for publication.

[31] Identification of Bacterial Class I Accessible Proteins by Disseminated Insertion of Class I Epitopes

By DOLPH ELLEFSON, ADRIANUS W. M. VAN DER VELDEN, DAVID PARKER, and FRED HEFFRON

The immune system is alerted to danger by the presentation of complexes on the surface of the infected cell. In the case of the mouse, these complexes are composed of antigens derived from the pathogen and specialized proteins of the major histocompatibility complex (MHC). Two sepa-
rate pathways, MHC I and MHC II, each drive cellular and humoral immune responses, respectively. In general, antigens presented in the context of MHC I are either derived from cytoplasmic proteins or, in the case of some professional antigen-presenting cells, via an alternate pathway through a lysosomal compartment.\textsuperscript{1,2} Antigens presented in the context of MHC II are generally derived via pinocytotic or phagocytic mechanisms.\textsuperscript{1}

Of the many pathogenic bacteria capable of mediating disease in humans and animals, one subset, intracellular pathogens, present unique challenges to researchers attempting to understand bacteria/host cell interactions. Intracellular pathogens are divided into two groups: those that reside within a phagolysosomal compartment (Salmonella sp., Mycobacterium tuberculosis, etc.) and those that reside within the cytoplasm (Listeria monocytogenes, Shigella sp., etc.). Intracellular pathogens adapt to their host cell environment by the selective secretion of proteins designed to alter the normal structural and metabolic machinery of the host cell, thus promoting bacterial survival and avoidance of host immune surveillance. Both phagolysosomal and cytoplasmic intracellular pathogens secrete proteins known to mediate their effects specifically within the host cell cytoplasm.\textsuperscript{3–5} Because cytoplasmic localization of the bacterial protein also infers access to the degradative machinery of the host cell proteosome, we have labeled these proteins class I (MHC, HLA) accessible proteins (CAPs). The identification of CAPs has proven laborious and imprecise, as whole genome analysis is of limited value in assigning a function to proteins encoded by bacterial genes with no known homology. Because a substantial proportion of open reading frames derived from whole genome analysis have no known function, a system that allows the identification of CAPs secreted in response to host cell interactions would be an invaluable tool for understanding the many levels of pathogen/host cell interactions.\textsuperscript{6} The identification of CAPs may enable the design of better bacterial carrier vaccines and identify whole new classes of potentially useful vaccine target proteins from different pathogens.

To identify Salmonella typhimurium CAPs potentially targeted to the host cell cytoplasm, we developed a resolvable Tn5-based transposon, which randomly distributes the MHC I (H-2K\textsuperscript{b}-restricted) ovalbumin epitope, SIINFEKL, throughout the bacterial chromosome. Epitope-tagged CAPs released from the infecting bacteria are processed by the proteolytic ma-

\textsuperscript{1} L. A. Morrison et al., J. Exp. Med. 163, 903 (1986).
chinery of the host cell and the carried ovalbumin epitope SIINFEKL is presented in the context of H-2Kb on the surface of the host cell. The approach, termed "disseminated insertion of class I epitopes (DICE), contains several inherent strengths in the identification of this important subset of bacterial proteins. First, DICE selection is conditional, and host class I-accessible proteins are isolated as a consequence of being processed and presented in the context of H-2Kb. Second, only in-frame insertions, which do not alter secretory signals, can be recovered. Third, the selection is simple and powerful; interesting strains are recovered quickly from a large population of infected cells by flow cytometry. Fourth, the selection is specific; bacteria cannot be recovered from macrophages that have presented SIINFEKL from nonsecreted intracellular proteins derived by bacterial attrition within in the phagolysosome because these bacteria would not be viable. Fifth, because the DICE transposon encodes a 6× histidine tag, the subcellular location of the protein can be visualized by microscopy, thereby enabling functional and phenotypic inferences to be drawn about proteins with no known homology. Sixth, the protein can be assessed readily as an epitope carrier by attenuating the bacterial strain and immunizing the appropriate animal model. Finally, genes encoding pathogen proteins identified by DICE can be cloned and their protein products assessed for their ability to engender protective responses in an immunized host.

Tn5-DICE Design

Transposition and Resolution of Tn5-DICE

Two primary events are involved in the successful insertion of class I epitopes in the chromosome of Salmonella: (1) transposition of the complete mobile genetic element and (2) resolution of an excessive transposon sequence to create a phenotypically distinguishable fusion protein. This system differs from other mutagenesis procedures in that it confers a phenotype in the host cell rather than in the mutated bacterium. Although the transposon is capable of insertion in all six frames, out-of-frame resolved insertions cannot be distinguished because the selection conditions dictate that any insertion must allow read-through of the class I epitope. In addition, sequences critical to preserving the bacterial proteins' trafficking pattern within the host cell must therefore be preserved. Because in-frame insertional events within a gene occur approximately 16% of the time, this system requires a transposon capable of frequent and indiscriminate distribution.

Tn5 satisfies this requirement because of its promiscuous insertion site preference and its high insertional frequency.\textsuperscript{8}

**Tn5 Transposon**

Wild-type Tn5 is a composite transposon that utilizes a conservative strategy as its principal mechanism of transposition.\textsuperscript{9} Two nearly homologous insertion sequences, IS50L and IS50R, flank antibiotic resistance genes in wild-type Tn5. IS50R contains transposase genes, and IS50L contains an ochre codon, rendering its sequences inactive.\textsuperscript{10} IS50L and IS50R are flanked by two 19-bp ends termed the inside end (I) and the outside end (O). Despite the presence of two insertion sequences in Tn5, functional mini-Tn5 variants have been constructed using only IS50R encoding the transposase, a resistance marker, and the I and O ends.\textsuperscript{11}

We surmised that we could take advantage of CAP access to the class I antigen processing and presentation machinery of the host cell to identify the genes encoding these proteins. If the genes can be engineered to carry an appropriate MHC I-restricted epitope, the *Salmonella* mutant strain could be isolated from a large pool and the gene encoding the CAP identified. Although the total number of CAPS encoded by *Salmonella* is unknown, we hypothesized that they must represent a small portion of the total *Salmonella* proteins. Because Tn5 integration is random and therefore capable of insertion in any of six frames, only in-frame-resolved insertions (1/6 of total) would be discernible. Based on these assumptions, we constructed a library of independent epitope insertions large enough to encompass every open reading frame. The high rate of Tn5 transposition, coupled with its lack of insertion site preference, made it the ideal mutagenesis system for this task.

The Tn5-DICE minitransposon consists of a Tn5-transposase and a kanamycin cassette flanked at its 5' and 3' ends by direct repeats of a minimal \textit{loxP} recombination site (Fig 1).\textsuperscript{12} The 5' end of the transposon consists of the Tn5 I end, the H-2K\textsuperscript{b}-restricted epitope SIINFEKL, a 6× histidine tag, and one \textit{loxP} site. Tn5-DICE is constructed such that on induction of Cre recombinase, the insertion is resolved at \textit{loxP} sites. The kanamycin and Tn5-transposase cassettes are segregated to nonreplicating loops and lost. When the insertion is in frame to a gene, the 49 amino acid-

FIG. 1. Tn5-DICE transposon. The minitransposon consists of kanamycin and transposase cassettes flanked by direct repeats of the minimal \textit{loxP} recombination site. The entire Tn5-DICE minitransposon is flanked by the IS50R \textit{I} and \textit{O} ends. The \textit{I} end, the H-2K\textsuperscript{b}-restricted ovalbumin epitope SIINFEKL, a 6× histidine site, and \textit{loxP}_{1} are translationally in frame.

resolved product creates a fusion protein carrying the SIINFEKL epitope (Fig. 2). \textit{Salmonella} proteins that end up in the cytoplasm of the host cell are processed and the SIINFEKL epitope is presented in the context of H-2K\textsuperscript{b}. The \textit{Salmonella} strain containing the resolved insertion is isolated

FIG. 2. In-frame resolution of Tn5-DICE. A \textit{Salmonella typhimurium} transconjugant carrying the Tn5-DICE transposon on the F plasmid was used as a donor to generate an insertional library (approximately 120,000 random insertions) in \textit{S. typhimurium} wild-type strain ATCC 14028 via P22 (HTint). Arabinose-induced expression of \textit{cre} recombinase resulted in the resolution of Tn5-DICE at \textit{loxP} sites, resulting in a resolved 49 amino acid product. Resolved in-frame insertions of the transposon result in expression of fusion proteins containing SIINFEKL and a 6× histidine tag.
Identification of CAP Genes

Materials

Strains: F\textsuperscript{+} *Eschericia coli* (Kan\textsuperscript{s}, Cam\textsuperscript{s}, Nal\textsuperscript{s}), *Salmonella typhimurium* (14028, American Type Culture Collection), *E. coli* S17\textsubscript{pir},\textsuperscript{14} and P22 phage

Reagents: Phycoerythrin (PE)-conjugated streptavidin

Antibodies: Biotinylated 25-D1.2 monoclonal antibody and FITC-conjugated anti-H-2D\textsuperscript{b}

Media: Luria broth (LB) agar with appropriate antibiotic(s) (P22 transductants are selected on LB agar plates containing 30 \(\mu\)g/ml kanamycin), RPMI 1640, and fetal bovine serum (FBS)

Mice: C57Bl/6 (H-2\textsuperscript{b})

Cells: L929 (Murine fibrosarcoma, American Type Culture Collection)

Equipment: CO\textsubscript{2} incubator and fluorescence-activated cell sorter

Methods

Transfer of Tn5-DICE to F\textsuperscript{+}

The Tn5-DICE transposon is transposed onto an F\textsuperscript{+} plasmid using a cotransfer mating selection.

1. An *E. coli* donor strain, which contains both an F\textsuperscript{+} plasmid and the Tn5-DICE-bearing plasmid, pDE510 (*tra\textsuperscript{+}lmo\textsuperscript{b}*-), is mated with a nalidixic acid-resistant *S. typhimurium* strain.

2. Nalidixic acid and kanamycin-resistant *Salmonella* transconjugants, which now contain F\textsuperscript{+}:Tn5-DICE, are confirmed by a P22 sensitivity test and the ability to transfer the transposon kanamycin marker back into *E. coli* or *Salmonella* recipients at a frequency equal to F\textsuperscript{+} plasmid transfer frequencies.

3. The *Salmonella*-specific bacteriophage, P22, is then used to make a lysate of the *Salmonella* strain containing F\textsuperscript{+}:Tn5-DICE. Because there is no sequence homology to F\textsuperscript{+} in *Salmonella*, the P22 phage lysate can be used to mutagenize a second *Salmonella* recipient (*Salmonella* strain containing CAPs by DICE 521 by fluorescence-activated cell sorter (FACS) analysis using the H-2K\textsuperscript{b}/SIINFEKL-specific monoclonal antibody 25-D1.2 as a marker.\textsuperscript{13}

\textsuperscript{13} A. Porgador et al., *Immunity* 6, 715 (1997).

\textsuperscript{14} S. A. Kinder et al., *Gene* 136, 271 (1993).
FIG. 3. Plasmids used for DICE analysis. (A) Plasmid carrying Tn5-DICE-resolvable minitransposon. (B) Arabinose-inducible Cre recombinase plasmid pBAD33cre.

pBAD33cre). The lack of F' homology in the recipient ensures that kanamycin-resistant transductants are derived as a result of transposition rather than homologous recombination. Transductants are selected by kanamycin resistance (30 μg/ml) on Luria agar. The Cre recombinase in pBAD33cre is under tight regulatory control of the pBAD promoter and mediates resolution and loss of the kanamycin resistance gene and the Tn5 transposase gene only when the strain is grown in the presence of arabinose (1 mM). The pBAD33cre plasmid is unstable and is lost in 3–10 generations when Salmonella strains bearing this plasmid are grown without selection (Fig. 3).15

4. The pool of S. typhimurium mutants is enriched for in-frame insertions of the resolved Tn5-DICE transposon within genes encoding secreted effector proteins by FACS as discussed later.

Identification of Strains Containing DICE Insertions by FACS Analysis

Assuming random integration of Tn5-DICE, approximately 1/6 (20,000) mutants of the 120,000 independent Tn5-DICE insertions we generated

should contain resolved in-frame insertions. Of this number, many insertions will be in metabolic genes that may be essential. In addition, many insertions will be contained in promoter or noncoding intergenic regions. Of the remaining mutants, far fewer will be contained within CAPs. The precise number of CAPs in \textit{S. typhimurium} is unknown. Because DICE insertions within CAPs may be rare events, a sensitive selection procedure was required. With the appropriate cell marker, FACS enables the isolation of extremely rare mutants.

\textbf{FACS Analysis of DICE Mutants in Murine Bone Marrow-Derived Macrophages}

1. Femurs are harvested from 4- to 6-week-old C57Bl/6 mice (H-2Kb). The femurs are scraped of muscle and connective tissue and are washed with RPMI, and bone marrow is exposed by removing proximal and distal ends with a scalpel.

2. Bone marrow cells are extracted by lavaging each end of the femur with a 3-cc syringe containing a 30-gauge needle and 2 ml of RPMI.

3. Bone marrow cells are then washed three times with RPMI at 37\(^\circ\) and resuspended at a density of \(1 \times 10^6\) cells/ml in RPMI 1640/10\% FBS containing 20\% L929 medium as a source of granulocytomacrophage colony-stimulating factor (GM/CSF). L929 medium is derived by harvesting L929 media 7 days after growing cells to confluence.\(^{16}\)

4. The cultures are allowed to differentiate to bone marrow-derived macrophages (BMDM) by culturing the bone marrow cells for 6 days at 37\(^\circ\)/5\% CO\(_2\).

5. BMDM are prepared for \textit{Salmonella} infection and FACS analysis by scraping the cultures and resuspending them in RPMI 1640/10\% FBS in a six-well plate at a density of \(1 \times 10^7\) cells per well.

6. The pooled library is grown overnight in Luria broth at 37\(^\circ\) with shaking.

7. The pooled DICE library is washed three times in RPMI 1640 and suspended in RPMI at a density of \(5 \times 10^8\) cells/ml.

8. Twenty microliters of the resuspended library is dispensed into individual wells of adhered BMDM (MOI = 1). An MOI of 1 or less limits multiple infections within the same BMDM. Typically, a 1\% infection rate is expected for \textit{S. typhimurium in vitro}.

9. The cultures are centrifuged for 2 min at 200 rpm to initiate contact and are incubated at 37\(^\circ\) for 1 hr.

10. After 1 hr, the cultures are removed and washed three times with phosphate-buffered saline (PBS, pH 7.4) preheated to 37\(^\circ\).

11. The cultures are then overlayed with 3 ml of RPMI 1640/10% FBS containing 50 μg/ml of gentamycin to kill extracellular bacteria and are incubated at 37° for an additional 2 hr.

12. The cultures are then washed three times with PBS preheated to 37°.

13. The cells are scraped from the plate, resuspended in 10 ml of RPMI 1640/1% FBS, and incubated on ice.

14. The cells are labeled for FACS analysis by incubating with FITC-conjugated anti-H-2D^b and biotinylated anti-H-2K^b/SIINFEKL (5 μg, 25-D1.2.13)

15. The cells are washed three times in PBS (4°) and incubated with 1 μg PE-conjugated streptavidin.

16. BMDM infected with the Salmonella-DICE library are sorted by first gating on the forward and side scatter population characteristic for macrophages. Bright red (PE-anti-H-2K^b/SIINFEKL) and bright green (FITC-conjugated anti-H-2D^b) populations, visualized in the double-positive quadrant, are sorted into a 5-ml polypropylene tube containing 2 ml of RPMI 1640/1% FBS.

17. The sorted cells are isolated by centrifugation and lysed in LB/1% Triton X-100.

18. The lysed cells are plated on LB agar and incubated at 37° overnight to recover Salmonella-DICE strains.

19. Infected BMDMs lacking CAP insertions may be recovered as a consequence of aggregate formation in the flow-sorted population. To ensure that recovery was to phenotypic expression of H-2K^b/SIINFEKL, the recovered bacterial colonies are counted, pooled, and subjected to two additional rounds of FACS sorting to enrich for Salmonella mutants containing CAP insertions. Individual isolates are then subjected to an additional round of FACS analysis to confirm their phenotype.

Sequencing of CAP Genes

In an effort to determine the identity of CAPs containing in-frame SIINFEKL insertions, we attempted to clone flanking DNA sequences by polymerase chain reaction (PCR). However, due to the small target sequence of the resolved Tn5-DICE transposon (147 bp), PCR strategies were prone to nonspecific primer annealing. We therefore opted to construct a unique system that allows specific and efficient identification of CAP genes (Fig. 4). In addition, this system was used to efficiently retransduce Tn5-DICE mutants and reconfirm their phenotypes. A KpnI–SacI fragment of a plasmid carrying the resolved Tn5-DICE transposon (pAV353a) was cloned into an ampicillin-resistant suicide vector (pGP704)\(^{17}\) to yield plas-

Fig. 4. Sequencing of Tn5-DICE-resolved CAPs. (A) Suicide plasmid pAV353, containing a resolved copy of Tn5-DICE, is conjugated into a naladixic acid-resistant, Cre-expressing Tn5-DICE mutant. (B) An ampicillin- and naladixic acid-resistant transconjugant is obtained via Cre-loxP recombination. (C) Isolated chromosomal DNA is restricted with EcoRI or Sall to clone 5' or 3' sequences flanking the original SIINFEKL insertion, respectively. Religated chromosomal fragments carrying the integrated plasmid pAV353 form functional replicons when transformed into E. coli S17Apir. Plasmid DNA from ampicillin-resistant transformants is isolated and analyzed using the Tn5-DICE-specific primer 5'-GCGGATATCCACCAC-3' (Sall) or 5'-TATGCCCGGGCCGTGGTGTTG-3' (EcoRI).
mid pAV353. This plasmid (pAV353) was transformed into *E. coli* S17λpir<sup>14</sup> and conjugated into spontaneous naladixic acid-resistant CAP mutants containing pBAD33cre. Site-specific integration of plasmid pAV353 at the chromosomal *loxP* site was selected following induction of the Cre recombinase by selecting naladixic acid- and ampicillin-resistant transconjugants. Chromosomal DNA was isolated, digested for 2 hr at 37° with one of several possible restriction endonucleases (see Fig. 4A, pAV353), and ligated overnight at 15°. Religated chromosomal fragments carrying the integrated plasmid pAV353 form functional replicons in *E. coli* S17λpir and carry either 3' (i.e., *SalI*) or 5' (i.e., *EcoRI*) sequences flanking the original SIINFEKL insertion (Fig. 4). Ampicillin-resistant transformants were analyzed further using the Tn5-DICE-specific primer 5'-GCGGATATCCACCCACCACC-3' (*ClaI*, *SalI*, *XhoI*, or *KpnI* digests) or 5'-TATGCCGG GTGGTGTTG-3' (*EcoRI*, *SacI* digests).

**Sequencing Protocol**

1. An ampicillin-resistant, nalidixic acid-sensitive donor strain (*E. coli* S17λpir) containing suicide plasmid pAV353 (amp<sup>r</sup> tra<sup>+</sup> mob<sup>+</sup>) is mated to a spontaneous nalidixic acid-resistant, Cre-expressing *S. typhimurium* Tn5-DICE mutant.

2. Transconjugants (amp<sup>r</sup> nal<sup>r</sup>) that carry an integrated copy of plasmid pAV353 at the chromosomal Tn5-DICE insertion site are selected.

3. Chromosomal DNA is prepared and restricted with either *EcoRI* or *SalI* (2 hr, 37°) to clone either 5' or 3' DNA sequences flanking the original SIINFEKL insertion, respectively. Digested DNA is absorbed over a DNA purification column to remove the restriction endonuclease and is ligated overnight (15°).

4. On transformation into *E. coli* S17λpir, religated circular fragments that contain the plasmid pAV353 form functional replicons, resulting in ampicillin-resistant transformants.

5. Plasmid preparations from ampicillin-resistant colonies are sequenced using the Tn5-DICE-specific primer 5'-GCGGATATCCACCACCACCACC-3' (*SalI* digest) or 5'-TATGCCGG GTGGTGTTG-3' (*EcoRI* digest).

**Tn5-DICE: Other Uses**

Although we have described a system for the use of the resolvable transposon in which CAPs are identified by incorporation of a classical class I epitope, the transposon has a range of other possible uses. This transposon has been engineered to accept a variety of different elements. For instance, we are using a modified version of Tn5-DICE to identify
Salmonella proteins that cycle into the class II (MHC, HLA) pathway. In addition, we are investigating the ability of the transposon to insert green fluorescence protein into genes, thus enabling us to identify in vivo-expressed genes by flow-sorting tissue homogenates. The transposon could also be used to modify vaccine carrier strains of Salmonella to augment or skew the immune response to the carried antigen by delivering eukaryotic effector proteins such as Jak2 or Tyk2 as CAP fusions. Finally, mutants generated by the transposon could be used to identify tissue-specific Salmonella CAPs, potentially useful proteins for regulating the timing of the immune response to carried antigens and thus generate immune responses more amenable to the life cycle of different pathogens.

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[32] *Bordetella pertussis* Adenylate Cyclase Toxin: A Vehicle to Deliver CD8-Positive T-Cell Epitopes into Antigen-Presenting Cells

By Pierre Guermonprez, Catherine Fayolle, Gouzel Karimova, Agnes Ullmann, Claude Leclerc, and Daniel Ladant

Introduction

Cytotoxic T lymphocytes (CTL) play a key role in the elimination of cells infected by virus or bacteria, as well as tumor cells that express particular epitopes (tumor associated antigens). This subset of T cells, which express the cell surface glycoprotein CD8, recognizes short peptides (the so-called CD8⁺ T-cell epitopes) associated with major histocompatibility complex (MHC) class I molecules. Although CD8⁺ T cells secrete cytokines (such as interferon-γ), their main role is to lyse cells that present the relevant peptide–MHC I complexes. Because MHC class I associated peptides are mostly derived from endogenously synthesized proteins, exogenous anti-