Attenuated Shigella as a DNA Delivery Vehicle for DNA-Mediated Immunization

Donata R. Sizemore,* Arthur A. Branstrom, Jerald C. Sadoff†

Direct inoculation of DNA, in the form of purified bacterial plasmids that are unable to replicate in mammalian cells but are able to direct cell synthesis of foreign proteins, is being explored as an approach to vaccine development. Here, a highly attenuated Shigella vector invaded mammalian cells and delivered such plasmids into the cytoplasm of cells, and subsequent production of functional foreign protein was measured. Because this Shigella vector was designed to deliver DNA to colonic mucosa, the method is a potential basis for oral and other mucosal DNA immunization and gene therapy strategies.

Department of Bacterial Diseases, Division of Communi
cations Diseases and Immunology, Walter Reed Army
Institute of Research, Washington, DC 20307–5100, USA.

*To whom correspondence should be addressed.
E-mail: dr.donata.sizemore@wrsmtp-cmcmail.army.mil
1Present address: Merck Research Laboratories, Post
Office Box 4, BLS-3, West Point, PA 19486, USA.

Shigella to enter epithelial cells and escape the phagocytic vacuole as a method for directing plasmid DNA to the cytoplasm of the host cell for protein synthesis and processing for antigen presentation (2). To attenuate the Shigella vector, we made a deletion mutation in the aid gene encoding aspartate β-semialdehyde dehydrogenase, an essential enzyme that is required to synthesize the bacterial cell wall constituent.
diaminopimelic acid (DAP) (3, 4). The resultant 15D construct, a Δasd isolate of Shigella flexneri 2a strain 2457T, was able to maintain the eukaryotic expression vector pCMVβ (5) in the absence of antibiotic-selective pressure. The plasmid pCMVβ expresses Escherichia coli β-galactosidase under the control of the immediate early promoter and enhancer from the human cytomegalovirus (CMV) in mammalian cells; this permitted easy analysis of mammalian cell-mediated gene expression after delivery.

Strain 15D was screened to ensure that the large plasmid that is essential for bacterial invasion of mammalian cells had not been lost during the genetic manipulations. Immunoblots verified that the strain continued to express the invasion-associated LpaB and LpaC polypeptides (6) and thus showed no loss of the invasion plasmid. To confirm earlier observations, we tested 15D and 15D(pCMVβ) for the ability to invade cultured baby hamster kidney (BHK) cells with and without DAP supplementation during the 90 min allowed for invasion (7–9). Examination by light microscopy of fixed and stained chamber slides revealed that in the absence of DAP, 15D and 15D(pCMVβ) entered 13% and 10% of the cultured BHK cells, respectively. By contrast, 33% (15D) and 29% (15D(pCMVβ)) of the BHK cells contained bacteria when DAP was present during the invasion step. Although both constructs were able to invade BHK cells, the addition of DAP during the invasion step increased the number of BHK cells infected and the number of viable bacteria recovered (9).

To test the ability of 15D to deliver plasmid DNA, we followed intracellular bacterial viability and β-galactosidase activity (Fig. 1) over a 48-hour time course (8, 10). Initially, 1 × 10^7 to 3 × 10^7 viable bacteria of each strain were recovered from monolayers of BHK cells with no detectable β-galactosidase activity in cell extracts. No β-galactosidase activity could be detected in bacterial extracts that were equivalent to the total number of bacteria added. At each assay point, a loss of 1 to 1.5 log units of viable bacteria occurred with no notable difference between strains 15D and 15D(pCMVβ). However, at both the 24- and 48-hour assay points, increasing units of β-galactosidase activity were readily detected in extracts of BHK cells infected with 15D(pCMVβ). The detected β-galactosidase activity did not result from expression within the bacteria because, although no activity was measured at the first two assay points, large numbers of viable bacteria were present. In addition, an isolate of 15D(pCMVβ) that did not express LpaB and LpaC (as measured by immunoblotting) was unable to bring about β-galactosidase activity at the 24-hour assay point.

Infected monolayers of BHK cells were immunostained to examine β-galactosidase expression within individual cells (Fig. 2) (8, 11). No intracellular immunostaining was observed in monolayers infected with...
either strain at the 30-min assay point (Fig. 2B). Only slight intracellular immunostaining was detected at the 4-hour assay point in monolayers infected with 15D(pCMVB) (Fig. 2, C and D). By the 24- and 48-hour assay points, positive immunostaining of several cells per field was observed in monolayers infected with 15D(pCMVB) (Fig. 2, E and F). Staining throughout the cytoplasm suggested that the plasmid DNA had been released from the bacterium into the cell cytoplasm, leading to transcription and translation by the mammalian cell. Immunostained cells also appeared to be rounded, possibly because of the presence of a large quantity of β-galactosidase protein. As measured by fluorescence-activated cell sorter (FACS) analysis, 1 to 2% of 5000 15D(pCMVB)-infected BHK cells expressed β-galactosidase at the 24-hour assay point (8, 10).

Visual examination of Leukostat-stained chamber slides of 15D(pCMVB)-infected BHK cells indicated that 28% of the cells contained one to five visually intact bacterial cells, with 1.7% containing five bacteria (Table 1). Four hours after gentamicin treatment, 26% of the cells contained visually intact bacteria, with <1% of the cells containing four bacteria. Therefore, invasion with one to five bacteria was required for foreign gene expression. Because pCMVB is a 7164-base pair plasmid that occurs in ~500 copies per bacterial cell, each bacterium is estimated to contain ~3.93 × 10^{-9} \mu g of DNA. Thus, intracytoplasmic delivery of no more than 4 × 10^{-9} to 20 × 10^{-9} \mu g of DNA by Shigella was sufficient for expression of β-galactosidase.

To demonstrate that gene delivery was not restricted to BHK cells, we infected murine P815 cells that express H-2d class I major histocompatibility complex (MHC) molecules with 15D(pCMVB). As shown in Table 2, 56.25 units of β-galactosidase activity were detected in lysates from P815 cells infected with 15D(pCMVB). Further experiments will be necessary to determine whether these cells can present Shigella-delivered DNA-encoded foreign antigens in the context of class I.

Studies of the ability of 15D to deliver plasmid DNA in vivo have begun in two small animal models, the guinea pig keratoconjunctival and murine intranasal models, which are used to study Shigella pathogenicity and immunobiology (12, 13). To determine whether 15D could deliver pCMVB to the ocular surface of the guinea pig eye, we stained corneas for β-galactosidase activity and visually examined them at various times after inoculation (12). Varying amounts of staining were observed in the outer region of the cornea near the sclera of the right eyes that received 15D(pCMVB), except those from day 8, in which staining was detected in only one of three corneas. Several areas typical of the staining observed in corneas that received 15D(pCMVB) are shown in Fig. 3B. No apparent endogenous β-galactosidase activity was detected in eyes inoculated with 15D. Histology experiments will be needed to examine in greater detail the percentage of cells and cell type(s) invaded by 15D(pCMVB) and those staining positive for β-galactosidase. In an initial experiment, spleen cells from intranasally inoculated BALB/c mice showed a moderate proliferative response to β-galactosidase protein (2.5 \mu g/ml) (13, 14). The stimulation index (14) was 3.6 when the inoculum was supplemented with DAP compared with 2.1 in the absence of DAP. Although preliminary, these experiments indicate that bacteria can be used to deliver plasmid DNA in vivo.

Our method for delivering functional DNA inside cells need not be restricted to Shigella because the invasion genes used by Shigella can be inserted into other bacteria such as E. coli (15). Likewise, other bacteria such as Listeria are able to invade cells and break out of the phagocytic vacuole into the cytoplasm (16). Although we have no formal proof that such a release of bacteria from the phagocytic vacuole into the cell cytoplasm is essential for DNA delivery, preliminary experiments with Salmonella typhymurium, which reaches the cytoplasm only with difficulty, suggest that this organism is not an efficient DNA delivery vehicle (17).

Any bacterial DNA delivery system will need to strike a balance between cell inva-

---

Table 1. Percentage of BHK cells infected and number of bacteria per BHK cell, as shown by microscopic examination. Chamber slides and bacteria were prepared as described (8). At least 400 BHK cells of each group were examined.

| Elapsed time (hours) | BHK cells infected (%) (mean) | Bacteria per infected BHK cell (mean ± SD) | Number of BHK cells containing 1 to 6 bacteria |
|---------------------|-------------------------------|-------------------------------------------|-----------------------------------------------|
|                     |                               |                                           | 1  | 2  | 3  | 4  | 5  | 6  | Total  |
| Strain 15D          |                               |                                           | 1  | 2  | 3  | 4  | 5  | 6  |        |
| 0.5                 | 39                            | 1.84 ± 1.2                               | 96 | 47 | 14 | 14 | 3  | 3  | 170     |
| 4                   | 36                            | 1.66 ± 0.94                              | 106| 36 | 13 | 5  | 0  | 1  | 161     |
| 24                  | 3.7                           | 1                                        | 17 | 1  | 2  | 2  | 0  | 0  | 17      |
| 48                  | 2.2                           | 1                                        | 10 | 1  | 2  | 2  | 0  | 0  | 10      |
| Strain 15D(pCMVB)   |                               |                                           | 1  | 2  | 3  | 4  | 5  | 6  |        |
| 0.5                 | 28                            | 1.35 ± 0.72                              | 76 | 29 | 7  | 5  | 2  | 0  | 119     |
| 4                   | 26                            | 1.40 ± 0.74                              | 96 | 16 | 4  | 1  | 0  | 0  | 116     |
| 24                  | 3.3                           | 1                                        | 14 | 1  | 2  | 2  | 0  | 0  | 15      |
| 48                  | 3.8                           | 1                                        | 18 | 1  | 2  | 2  | 0  | 0  | 19      |

Fig. 3. Ability of 15D to deliver pCMVB to ocular tissue. (A) Left cornea (15D) and (B) right cornea (15D(pCMVB)), 48 hours after ocular inoculation. Arrowheads indicate areas of β-galactosidase staining.

Table 2. β-Galactosidase activity in P815 cells after infection with 15D(pCMVB). Bacteria used to infect P815 cells were grown as described (8). After addition of the bacterial cultures containing DAP to the nonadherent P815 cells cultured in six-well plates, the plate was spun at 500g for 5 min. Bacteria and P815 cells were allowed to interact for 90 min. The cells were then extensively washed with DMEM and resuspended in DMEM containing gentamicin (100 \mu g/ml) for a 1-hour incubation at 37°C in the presence of 5% CO_{2}. The cells were again extensively washed and resuspended in DMEM containing gentamicin (20 \mu g/ml) for overnight culture at 37°C in the presence of 5% CO_{2}. β-Galactosidase activity and protein concentrations were determined at 24 hours as described (8, 10).

| Source                  | β-Galactosidase (units per milligram of protein) |
|-------------------------|-----------------------------------------------|
| P815 cells              | 3.04                                         |
| P815 cells + 15D        | 5.62                                         |
| P815 cells + 15D(pCMVB) | 56.25                                        |
REFERENCES AND NOTES

1. J. P. Donnelly, J. B. Ulmer, M. A. Liu, J. Immunol. Methods 178, 1-10(1994).
2. N. H. Journier, M. C. Prévost, P. L. Sansonnens, EMBO J. 11, 1991 (1992).
3. K. Nakayama, S. M. Kelly, R. Curtis III, Biotechnol. 6, 693 (1998).
4. A detailed description of Shigella flexneri 2a strain 2457T was constructed by polymerase chain reaction (PCR) amplification of an E. coli asd gene (C. Haziza, P. Stragier, J. C. Ratte, EMBO J. 1, 379 (1992)) incorporated into a suicide vector (D. S. A. Reis, W. R. F. V. paper presented at the 33rd International Conference on Antimicrobial Agents and Chemotherapy, New Orleans, 20 October 1993). Positive clones were selected with the use of E. coli pG1037 (3). Reverse PCR amplification was used to identify 533 base pairs of the E. coli asd structural gene (positions 439 to 961) for forward primer, 5'-GATGCCTTCAACATCGTCCG-3', reverse primer, 5'-CGAGGGCTTATGCCCTTC-3'). The kanamycin resistance cassette from pC4K-KOX (Pharma) was digested with Sma I and cloned between the flanking asd sequences. A 2.5-kb fragment of the remaining asd gene with the internal kanamycin resistance cassette was PCR-amplified (forward primer, 5'-SacI-CCGATATTCGCGCCG-3'; reverse primer, 5'-SalI-CGGTATGTCGCCG-3') and cloned into SacI-Sal I site of the positive selection suicide vector pCD442 [M. S. Donenberg and J. B. Kaper, Infect. Immun. 59, 4310 (1991)], creating pSEAK. Clones were transfomed into SM10Apxr [R. Simon, U. Prefert, A. Puhlcr, Biotechnology 1, 784 (1983)] and selected for resistance to ampicillin. SM10Apxr(SEAK) was conjugated with S. flexneri 2a strain 2457T (C. Caskey) and conjugants were selected for resistance to ampicillin and tetracycline. PCR analysis of resistant isolates showed that plasmids integrating into the chromosomal DNA had recombinated with the downstream portion of the asd fragment of pSEAK. Growth on sucrose resulted in a second recombination event [J. Quaridt and M. F. Hynes, Gene 127, 15 (1993)], and isolate 15C was obtained by screening the resultant isolates for resistance to kanamycin and the requirement for DAP. Hybridization and PCR analysis confirmed a deletion in asd. Further, the mutated Shigella virulence determinants of each strain used to inoculate overnight LB broth cultures containing DAP (50 mg/ml) with or without DAP (50 mg/ml) added to mice of semi-confluent BHK cells (1 x 10^7) at 10:1. For flask assays, bacteria were washed in PBS, resuspended in PBS, and divided into two equal volumes. The mid-log phase cultures were diluted 1:50 and grown to an approximately mid-log phase in the presence of DAP. For chamber slide assays (Nunc) and 24-well plate assays, 200 ml of agarose gel balanced solution (HBB5) with or without DAP (50 mg/ml) was added to three wells of semi-confluent BHK cells (1 x 10^5) at 10:1. For flask assays, bacteria were cultured overnight in HBB5, washed in PBS, and divided into two equal volumes. The mid-log phase cultures were diluted 1:50 and 2 ml of the bacterial solution was added to a flask of BHK cells (1 x 10^7). In all assays, bacteria were allowed to interact with BHK cells overnight at 37°C in the presence of 5% CO_2. Nonadherent bacteria were removed by extensive washes with HBB5. Extracellular bacteria were then killed by the addition of Dubelloco's modified Eagle medium (DME) with 10% heat-inactivated fetal bovine serum (BioWhittaker) and gentamicin (50 mg/ml). For plating assays, 175.0.2% Triton X-100 solution and appropriate dilutions were plated on tryptic soy agar (TSA) containing DAP plates for determination of viable bacterial counts. At the indicated times, the plates were removed, extensively washed, fixed, and immunostained with a Leukostat kit (Fisher) before light or fluorescent microscopy. An Instar statistical program (Graphpad, San Diego, CA) was used to calculate means, SDs, and SEs. All data presented are representative of experiments performed two or more times.

9. The ability of a detailed description of S. flexneri 2a strain 2457T to invade mammalian cells in culture was assessed to examine the requirement of DAP during the adherence and invasion step. Bacterial solutions, with or without DAP were allowed to interact with SRK cells for 90 min, washed extensively, and then treated with gentamicin-containing media for 30 min before plating. In the absence of DAP, 10^7 ± 404 (150) and 10^6 ± 332 (150D(CMV)) viable bacteria were recovered, versus 8.2 ± 10^7 ± 1 x 10^7 (15D(CMV)) and 8.6 ± 10^7 ± 8.5 x 10^7 (15D(CMV)) when DAP was present [mean ± SE; P = 0.024 (Mann-Whitney test)].

10. For guinea pig inoculations, 7.2 ± 10^5 D5 beta in a 25-ml volume were placed on the left eye and 4 ± 10^5 D5(CMV) bacteria in a 25-ml volume were placed on the right eye, as described [A. B. Hartman, C. J. Powell, C. L. Schultz, E. V. Oaks, K. H. Eckels, Infect. Immun. 59, 4075 (1991)]. On days 1, 2, 6, and 8, animals were killed and each eye was removed for dissection of corneas and adjacent sclera. Whole eyes or dissected corneas were stained for β-galactosidase with [R. J. Sanes, J. L. R. Rubenstein, J. Nicolas, EMBO J. 9, 3133 (1990)]. After overnight development, corneas were examined under a dissecting microscope, scored for staining, and photographed. Corneas from three guinea pigs were examined on days 1, 2, and 8; corneas from two guinea pigs were examined on day 6. All animal experiments were conducted in accordance with the Guide for Care and Use of Laboratory Animals (NIH publication 99-23, National Institutes of Health, Bethesda, MD, 1985).

11. L. L. Van De Verg et al., Infect. Immun. 63, 1947 (1995).

12. D. R. Sizemore, A. B. Hartman, A. A. Branstrom, J. C. Sadow, in preparation. Female BALB/c mice received two intranasal inoculations of 1 (10^7 15D(CMV)) or 15D(CMV) in a 30-ml volume, 4 weeks apart. The stimulation index is the difference between the mean OD_590 values for β-galactosidase stimulation of 15D(CMV) cells and for unstimulated 15D(CMV) cells, divided by the difference of the mean OD_590 values for β-galactosidase stimulation of 15D(CMV) cells and for unstimulated 15D(CMV) cells (where OD_590 is the optical density at 590 nm).

13. P. J. Sansonetti et al., Infect. Immun. 39, 1392 (1983).

14. D. A. Portnoy and S. Jones, Ann. N.Y. Acad. Sci. 730, 15 (1994).

15. D. R. Sizemore, A. A. Branstrom, J. C. Sadow, unpublished data.

16. A. Zychlinsky, M. C. Prévost, P. L. Sansonnens, Nature 358, 167 (1992).

17. J. Sun, J. Holmgren, C. Czerkinsky, Proc. Natl. Acad. Sci. U.S.A. 91, 10795 (1994).

18. We thank C. Harrington, C. Caskey, II, E. V. Oaks, A. Hartman, and individuals in the Division of Medical Audio Visual Services at Walter Reed Army Institute of Research for providing materials or technical expertise. The views expressed in this report are those of the authors and not those of the U.S. Department of Defense.

19. 6 June 1995; accepted 14 September 1995.