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Transcutaneous immunization of healthy volunteers with an attenuated *Listeria monocytogenes* vaccine strain and cholera toxin adjuvant☆,☆☆

Elizabeth H. Eypper[a], Paul V. Johnson[a], Eva I. Purro[b], Elizabeth L. Hohmann[a,*]

[a] *Infectious Diseases Division, Massachusetts General Hospital, Boston, MA 02114, USA*
[b] *List Biologicals, Inc., 540 Division Street, Campbell, CA 95008, USA*

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ABSTRACT

Background: Attenuated *Listeria monocytogenes* vaccine strains have been administered intravenously (Le et al. [1], Maciag et al. [2]) and orally (Angelakopoulos et al. [3], Johnson et al. [4]) to humans. Here, one was given transcutaneously with cholera toxin adjuvant.
Methods: Eight healthy volunteers were studied (5 active, 3 placebo). Safety was assessed by physical exam and labs. Systemic immunological responses were measured by ELISA and IFN-gamma ELISpot.
Results: 4/5 active volunteers had cellular responses to listerial antigens. 5/5 active volunteers showed humoral responses to cholera toxin.
Conclusions: An attenuated *L. monocytogenes* vector was safely administered transcutaneously. Topical administration appeared at least as immunogenic as previously studied oral delivery.

1. Introduction

The skin contains a complete complement of the body's immune cells. An adult may have as many as 20 billion T cells of various subtypes in the skin, exceeding the number in circulation (reviewed in [5]). Dermal dendritic cells capture antigens and deliver them to the skin-draining lymph nodes and activate naïve or central memory T cells stimulating both local and systemic immune responses [5].

Intradermal injections can be vaccine-sparing and effective [6]. Intradermal delivery can be done using traditional needles, microneedles, air pressure insufflation, iontophoresis, scarification, or by simple absorption. The microbial enterotoxins cholera toxin and *Escherichia coli* heat labile toxin and mutants thereof have been shown to be mucosal and cutaneous immunogens and potent adjuvants. Transcutaneous administration of LT has enhanced immune responses to a co-administered bacterial protein [7], and an injected influenza vaccine [8].

Live attenuated bacterial vectors based upon enteric pathogens like Listeria and Salmonella species are relatively easily engineered to express foreign antigens. Unfortunately, they have not proved highly successful in human studies in stimulating immune responses to engineered heterologous antigens. Live bacterial vectors administered orally are plagued by several (at least theoretical) concerns: inadequate attenuation, reversion, recombination, shedding, and the potential for transmission. We previously evaluated a highly attenuated *Listeria monocytogenes* organism expressing an influenza A nucleoprotein antigen by the oral route in single escalating oral doses in volunteers, and found this organism safe, transiently shed, but poorly immunogenic and probably over-attenuated for oral delivery [4].

Despite this apparent failure, we hypothesized that this would be an ideal, highly attenuated organism to test transcutaneous application of a live attenuated enteric vector. In mice *L. monocytogenes* stimulates potent CD4 and CD8T cell responses and effectively delivers both viral and tumor antigens (reviewed in [9]). Besides vertical transmission in utero, *L. monocytogenes* is not transmitted person-to-person. Unlike oral delivery, cutaneous delivery (though not presentation or antigen processing) can be rapidly stopped by topical disinfection, and minimizes concerns related

Abbreviations: CBC, complete blood count; CEF, CMV, EBV, and Influenza virus; CFU, colony forming units; GMP, good manufacturing processes; IFN, interferon; LLO, listeriolysin; LT, *E. coli* heat labile toxin; PBMC, peripheral blood mononuclear cells; PHA, phytohemagglutinin; SFC, spot-forming cells.

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* Corresponding author at: Infectious Diseases Division, Massachusetts General Hospital, 55 Fruit Street, Boston, MA 02114, USA. Tel.: +1 617 724 7532; fax: +1 617 724 3761.
E-mail address: ehohmann@partners.org (E.L. Hohmann).

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to shedding. We included a native commercially available cholera toxin adjuvant, in an attempt to maximize immune responses. The project was proposed as a novel physiological study of a bacterial vector delivered transcutaneously, and *not* as development of a new influenza vaccine.

We show that cellular immune responses to complex listerial antigens can be engendered by the transcutaneous route. Though not compared "head-to-head" these results may be superior to those engendered by delivery of a single large dose orally.

2. Materials and methods

2.1. Bacterial strain

L. monocytogenes strain BMB72 expressing a secreted influenza A nucleoprotein antigen was derived from *L. monocytogenes* strain 10403S as described [4].

2.2. Human subjects

The study was reviewed and approved by the Partners IRB, the Harvard Institutional Biosafety Committee, the FDA (IND # 13937) and registered at CT.gov (NCT01311817). Healthy adults who were 18–55 years old and provided written informed consent underwent a complete medical screening by history, physical exam and laboratory evaluation as described [4]. Subjects were not screened for previous infection with *L. monocytogenes* or influenza and were paid per IRB norms. There is no clinically validated serological test for prior or active infection with *L. monocytogenes*.

2.3. Inoculum and administration

Research laboratory GMP methodologies and Standard Operating Procedures acceptable to the US FDA were used to grow and characterize bacterial inocula for clinical use. Bacteria from a master cell bank were grown aerobically with rotary shaking in 2 L glass flasks in trypticase soy broth (DIFCO, Sparks, MD) to optical density $1.0_{600\,nm}$, harvested by centrifugation, and re-suspended in normal saline/20% USP glycerol (15:1 concentration by volume). Bacteria were not washed. Individual cryovials were filled by manual pipetting, frozen at −80 °C, and assessed at manufacture and every 3–6 months thereafter, for purity and stability, using microbial limits testing and CFU/ml determinations respectively over time. There were pre-specified acceptance criteria for use in humans.

Native, sterile, preservative-free biologically active cholera toxin (List Biological Laboratories, Inc.) was used. Based upon spread plate cultures, the thawed inoculum contained 2.0×10^{10} CFU/dose and also contained approximately 2× that number of dead organisms. Cholera toxin (50 μg) was added to the bacterial inoculum (Lot ELH072910), and a 1 mL volume pipetted onto the absorbent pad of a Tegaderm patch (3 M Company) (Fig. 1). Preclinical cutaneous toxicology studies were performed using CT and the bacterial strain in animals by both the oral and cutaneous routes.

The deltoid regions were first exfoliated with dry ECG prep pads (Marquette Medical Systems, Jupiter, FL), then with electrode skin prep pads containing alcohol and silica (Dynarex Company) to remove excess stratum corneum. Vaccine patches were then applied and left in place for up to 24 h (Fig. 1). Each volunteer received two patches: either one active and one placebo (saline), or two placebos. Two patches were used, to assess reaction to the patch alone and the volunteer's assignment of active vs. placebo patch. Subjects were randomly assigned to active vs. placebo and the study physician and subjects were blinded to assignments. After removal, patch sites were sequentially cleaned with alcohol wipes, a surgical scrub sponge with 3% chloroxylenol (Beckton, Dickinson and Company), and Calstat antiseptic hand rub (Steris).

2.4. Clinical assessments

Subjects were seen as outpatients. Vaccinations were given on days 0, 21, and 35. Volunteers were examined 24 and 48 h after removal of patches. Safety labs (CBC, liver function tests) were done 48 h after each vaccine removal and at a final visit. Temperature was monitored at each visit, and by subjects at home. PBMC were isolated from heparinized blood on days 0, 35, and 42. Serum was collected weekly starting on day 21 (Supplementary Fig. 1, study schema; online).

2.5. Interferon-γ ELISpot studies

IFN-γ ELISpot studies were performed as described [3,4] using freshly isolated PBMC on Immobilon P (MAIPS4510; Millipore) plates containing various peptide pools and complex antigens. Bulk, freshly isolated mononuclear cells isolated by Ficoll gradient centrifugation and counted using a slide-based Nexcelcom automated cell counter were used without further characterization. Control wells included PHA and CEF, a commercial standard peptide pool including 32 CMV, EBV and influenza virus peptides 8–12 amino acids in length (AnaSpec). Test peptides included 3 influenza nucleoprotein peptide pools and a single LLO peptide pool used in our prior oral administration study [4], the latter a gift of Cerus corporation. Complex antigens included whole sonicated *L. monocytogenes*, and a soluble antigen from spent *L. monocytogenes* culture medium. Spots were counted by an automated reader (Immunospot 3; CTL). ELISpot results are presented as mean values of duplicate wells per condition as SFC/10^6 PBMC. A positive response was defined as more than 2-fold greater than baseline spots for that antigen and over 100 SFC/10^6 PBMC as in our earlier study [4]. Groups were compared using Fisher's exact test (two tailed).

2.6. Seroconversion/ELISA

Serum samples were studied by ELISA to quantify immunoglobulin G [10] directed against the complex listerial antigens, recombinant his-tagged LLO, cholera toxin, and recombinant influenza A nucleoprotein over time. Antigens (10 μg/mL) were used to coat Maxisorp 96-well plates (Nalge Nunc International). Assays were performed as described [4] with goat-anti-human IgG affinity-purified peroxidase-labeled antibody and read on a Vmax kinetic microplate reader (Molecular Devices). Endpoint titers are reported as the highest dilution at which a serum sample read at $\geq 0.15\ OD_{450\,nm}$, an empirically chosen cutoff value. Fourfold or greater increases in endpoint titer were considered a positive result. Groups were compared using Fisher's exact test (two tailed).

3. Results

3.1. Subjects

A total of 22 people were screened by phone, 12 underwent complete screening, and 8 completed the study (7 men, 1 woman; all Caucasian).

3.2. Clinical responses

No volunteer had fever, serious or unexpected problems or clinically significant abnormal laboratory findings. No subject at any time point studied had liver function tests outside the normal range (ALT, AST, alkaline phosphatase and Total and Direct Bilirubin).