Use of Ensure® nutrition shakes as an alternative formulation method for live recombinant Attenuated Salmonella Typhi vaccines

Karen E Brenneman1,3, Amanda Gonzales1, Kenneth L Roland1* and Roy Curtiss 3rd1,2

Abstract

Background: To be effective, orally administered live Salmonella vaccines must first survive their encounter with the low pH environment of the stomach. To enhance survival, an antacid is often given to neutralize the acidic environment of the stomach just prior to or concomitant with administration of the vaccine. One drawback of this approach, from the perspective of the clinical trial volunteer, is that the taste of a bicarbonate-based acid neutralization system can be unpleasant. Thus, we explored an alternative method that would be at least as effective as bicarbonate and with a potentially more acceptable taste. Because ingestion of protein can rapidly buffer stomach pH, we examined the possibility that the protein-rich Ensure® Nutrition shakes would be effective alternatives to bicarbonate.

Results: We tested one Salmonella enterica serovar Typhimurium and three Salmonella Typhi vaccine strains and found that all strains survived equally well when incubated in either Ensure® or bicarbonate. In a low gastric pH mouse model, Ensure® worked as well or better than bicarbonate to enhance survival through the intestinal tract, although neither agent enhanced the survival of the S. Typhi test strain possessing a rpoS mutation.

Conclusions: Our data show that a protein-rich drink such as Ensure® Nutrition shakes can serve as an alternative to bicarbonate for reducing gastric pH prior to administration of a live Salmonella vaccine.

Keywords: Salmonella vaccine, Gastric pH neutralization, Bicarbonate, Ensure nutrition shake, Low gastric pH mouse model

Background

Live recombinant attenuated Salmonella–vectored vaccines (RASV) have the potential to provide protection against a variety of human non-Salmonella pathogens at low cost. By using the Salmonella cells to express a heterologous protective antigen, RASVs can induce humoral and cellular immune responses directed at a pathogen of interest [1]. RASVs have the additional advantage of stimulating mucosal immune responses, due to their oral route of immunization. Oral delivery provides RASVs with the opportunity to invade and colonize the intestinal gut-associated lymphoid tissues (GALT), where they actively interact with the host immune system to stimulate robust humoral, mucosal and cellular immune responses [2]. To allow the vaccine cells to reach the intestinal tissues more rapidly, human subjects are frequently required to fast prior to immunization as a means to clear the gastrointestinal tract of food [3]. However, fasting also causes the gastric pH of humans to fall below 2.0 [4,5]. This poses a non-trivial challenge to the success of the immunization, as Salmonella species, particularly S. Typhi [6], are not particularly resistant to low pH (succumbing below pH 3.0), and the mutations necessary for attenuation in RASVs often impose additional sensitivity to acid [7-11]. Our lab has constructed RASV strains exhibiting regulated-delayed attenuation [12]. These S. Typhi-derived RASVs, χ9633(pYA4088), χ9639(pYA4088) and χ9640(pYA4088), are susceptible to a number of environmental stresses, including low pH [13]. To administer an acid-sensitive vaccine strain via the oral route, the vaccine must be given using a strategy that not only actively protects the vaccine cells...
from gastric acid, but also does not negatively affect vaccine viability or the development of an immune response following vaccination.

Most researchers address the problem of low gastric pH by administering an antacid such as sodium bicarbonate prior to the RASV [14-17]. The antacid rapidly neutralizes the gastric acid, allowing the vaccine cells to transit the gastric compartment under neutral or mildly acidic conditions [18,19]. This combination of a liquid RASV formulation with antacid is highly effective and promotes the development of protective immune responses [20,21]. However, bicarbonate is not without problems. In order to efficiently neutralize gastric acid, a surprisingly large volume of bicarbonate must be given, as gastric mixing is not efficient enough to thoroughly disperse small volumes of bicarbonate completely throughout the stomach [22,23]. In addition, bicarbonate has a rather unpleasant taste to most palates and efforts to improve this aspect will positively enhance the experience of the volunteers in a clinical trial or vaccinees receiving licensed vaccines. Flavoring agents are sometimes added to vaccine formulations for this reason [24].

In preparation for a clinical trial to assess the three RASV strains listed above, we wanted to investigate the administration of high concentrations of bicarbonate as an alternative to bicarbonate. Protein is capable of buffering gastric acid and raises the gastric pH within minutes of ingestion [25,26]. As a food-borne pathogen, Salmonella appears to take advantage of this gastric acid buffering during infection scenarios. In the presence of protein-rich food, the infectious dose of Salmonella is significantly lower than in the absence of food [27]. Thus, we hypothesized that the administration of protein, specifically Ensure® Nutrition shakes, immediately prior to and following immunization would provide the same protection from the low pH gastric environment as bicarbonate. Using Ensure® also provides a carrier with a taste likely to be more pleasant than bicarbonate for most vaccinees. We examined the survival of S. Typhi wild type and vaccine strains when suspended in Ensure® or a bicarbonate solution and how these compounds, when administered to mice with a low gastric pH, influenced survival during gastric transit.

Results
Survival of recombinant attenuated Salmonella Typhi vaccine strains in bicarbonate and Ensure®
To be an effective vaccine formulation, the carrier or co-administered substance must not negatively affect the viability of the vaccine cells. We monitored the effect of bicarbonate and Ensure® (milk chocolate flavor) on the viability of the three S. Typhi vaccine strains and model S. Typhimurium strain for four hours (Figure 1). Most of the S. Typhi strains, including χ9633, χ9639, ISP1820 and Ty2, and both of the S. Typhimurium strains we tested underwent a statistically significant increase in numbers when incubated in Ensure®, indicating that Ensure® could support the growth of these strains. Cell numbers of strains suspended in bicarbonate tended to decrease over time, but the decrease was statistically significant only for strain Ty2 (T0 vs T4, p = 0.035) (Figure 1B). Interestingly, there were no significant changes in cell numbers for strains χ8438 and χ9640 in either bicarbonate or Ensure® (Figure 1C). There were statistical differences in the numbers of cells recovered from Ensure compared to bicarbonate at the 2 and 4 h time points for a number of strains (Figure 1A, B, D), primarily due to the fact that Ensure® apparently supported the growth of these strains while bicarbonate did not. We also examined survival in vanilla and strawberry Ensure® and the flavor did not affect strain viability (data not shown).

Bicarbonate and Ensure® protect vaccine cells during low pH gastric transit
Another characteristic of an effective RASV delivery formulation is that it must protect cells from the low pH of the gastric environment. To examine the ability of bicarbonate and Ensure® to combat gastric pH, these were used to buffer the stomach pH of mice. Because the gastric pH of a fasted mouse is about pH 4.0 and the gastric pH of a fasted human is about pH 1-2 [4,5,28], gastric acid secretion was induced in mice by subcutaneous histamine injection (see Methods section) prior to immunization to better mimic the situation in humans. Using this protocol, the pH in the mouse stomach is reduced to around 1.5 [29]. Mice received either bicarbonate or Ensure® prior to and immediately following immunization. Control mice received no treatment. Vaccine viability was measured following gastric transit (Figure 2). Compared to the no treatment group, administration of Ensure® significantly increased the number of viable cells that reached the small intestine for two of the S. Typhi strains and for the S. Typhimurium strain (p = 0.0019 for χ9633(pYA4088), p = 0.0256 for χ9640(pY4088) and p = 0.0006 for χ9558 (pYA4088). This was a 599-, 75.0- and 647-fold increase, respectively, in the geometric mean number of viable cells to reach the ileum. Bicarbonate similarly improved the survival of χ9640(pYA4088) (p = 0.0190) and χ9558(pYA4088) (p = 0.0379) during gastric transit, resulting in a 41.0- and 8.79-fold increase in the geometric mean number of cells to reach the ileum, respectively. Administration of bicarbonate did not significantly impact the survival of χ9633 (pYA4088) or χ9639(pYA4088) (p = 0.2317 and 0.4945, respectively) compared to the no treatment controls.

The Ensure treatment was better than bicarbonate at increasing the gastric transit survival of strains χ9633.
Interestingly, the survival of strain χ9639(pYA4088) was not impacted by either Ensure or bicarbonate treatments (Figure 2B). Further, this strain survived gastric transit in mice that did not receive bicarbonate or Ensure® somewhat better than the other S. Typhi strains (Figure 2A, B, C) although the difference was not statistically significant (p = 0.06).

Discussion

The vast majority of clinical RASV trials have made use of sodium bicarbonate as a means to protect vaccine cells from low gastric pH. In fact, field trials with the licensed typhoid vaccine strain Ty21a demonstrated that the administration of bicarbonate produced a superior immune response as compared to other vaccine formulation strategies [30,31]. Our results are consistent with the idea that ingestion of a buffering substance prior to oral immunization promotes the survival of vaccine cells. The administration of bicarbonate prior to and immediately following immunization significantly improved the survival of both S. Typhi χ9640(pYA4088) and S. Typhimurium χ9558(pYA4088) during gastric transit. Interestingly, strain χ9640(pYA4088) was the most immunogenic, among the three S. Typhi strains tested here, in a recent clinical trial [32]. Sodium bicarbonate is generally regarded as safe, and has been shown to have no effect on the viability of wild-type Salmonella [33].

Our results demonstrated that high concentrations of protein administered before and after immunization can act as a substitute for bicarbonate. Ensure® provided a greater degree of protection from the gastric environment than bicarbonate for S. Typhi strain χ9633 (pYA4088, pWSK129) (Figure 2A) and S. Typhimurium strain χ9558(pYA4088, pWSK129) (Figure 2D), and provided protection equivalent to bicarbonate for S. Typhi strain χ9640(pYA4088, pWSK129) (Figure 2C). No effect of bicarbonate or Ensure® was observed for the rpoS Ty2 derivative, S. Typhi strain χ9639(pYA4088, pWSK129) (Figure 2B).

Neither sodium bicarbonate nor Ensure® was able to significantly increase the survival of χ9639(pYA4088)
during gastric transit. This is interesting, because of the four RASV strains tested in this study, χ9639 is the only rpoS mutant, due to the fact that parent strain Ty2 carries a mutation in rpoS [34]. Salmonella rpoS mutants are significantly more sensitive to low pH than strains with a functional RpoS because they are unable to sustain an acid tolerance response (responsible for protecting cells against low pH) for more than 20 minutes [7].

The problem may have been exacerbated by the presence of the ΔPfur81::TT araC PBAD fur mutation in χ9639, as Fur and RpoS jointly regulate induction of the acid tolerance response [7,35]. The amount of Fur present in a ΔPfur81::TT araC PBAD fur S. Typhi mutant is substantially lower than a wild-type strain, regardless of the arabinose concentration during growth and, with regard to survival at low pH, is indistinguishable from a fur deletion mutant (29). Note that strain χ9640 is also a derivative of Ty2, but in this strain, the rpoS gene has been replaced with a functional gene from ISP1820 (parent of χ9633). The S. Typhi murium strain χ9558 has a functional rpoS gene, since its parent is RpoS+. Thus, it is likely that a functional rpoS is required in order to benefit from bicarbonate and Ensure treatment, at least in this genetic background.

Conclusions

The Ensure® nutrition shake was able to act as a substitute for bicarbonate during oral inoculation to enhance bacterial survival during passage through a low gastric pH compartment. Ensure® provided protection better than or equivalent to bicarbonate for all of the strains tested. The failure of both Ensure® and bicarbonate to protect an rpoS mutant during gastric transit suggests that in future clinical trials, investigators should carefully evaluate the degree of protection necessary for the specific RASV strain being evaluated and perform a careful evaluation of the buffering agent used to neutralize gastric pH.

Methods

Bacterial strains, plasmids and culture conditions

The bacterial strains and plasmids used in this study are listed in Table 1. Strain χ9633 is derived from S. Typhi ISP1820, an RpoS+ strain. Strains χ9639 and χ9640 are derived from parent strain Ty2, which is RpoS−. Strain χ9640 was rendered RpoS+ by transduction [13]. For routine use, strains were propagated in LB medium (which contains 0.1% glucose) [36] supplemented with 0.05%
Table 1 Salmonella vaccine strains and plasmids used in this study

| Strain      | Salmonella Serovar | Genotype/Phenotypea | Reference |
|-------------|--------------------|---------------------|-----------|
| y9558       | Typhimurium        | Δprrt−2426 Δlfdgd−6126 ΔPrrt−6126 TT araC PBSD fur ΔPrrt−6126 TT araC PBSD c2 ΔaraE25 ΔaraBAD23 ΔlfdgpA198ΔaraC PBSD lac T Δlpar8125 Δgmdfcl81 Δserovar genotype/phenotype ΔaraC lac T Δlpar8125 Δgmdfcl81 Δserovar genotype/phenotype ΔaraC| [39] |
| y9633       | Typhi ISP1820      | ΔPrrt−6126 TT araC PBSD crap ΔPrrt−6126 TT araC PBSD fur Δprrt−2426 Δlfdgd−6126 ΔlfdgpA198ΔaraC PBSD lac T Δlpar8125 Δgmdfcl81 Δserovar genotype/phenotype ΔaraC lac T Δlpar8125 Δgmdfcl81 Δserovar genotype/phenotype ΔaraC| [13] |
| y9639       | Typhi Ty2          | ΔPrrt−6126 TT araC PBSD crap ΔPrrt−6126 TT araC PBSD fur Δprrt−2426 Δlfdgd−6126 ΔlfdgpA198ΔaraC PBSD lac T Δlpar8125 Δgmdfcl81 Δserovar genotype/phenotype ΔaraC lac T Δlpar8125 Δgmdfcl81 Δserovar genotype/phenotype ΔaraC| [13] |
| y9640       | Typhi Ty2          | ΔPrrt−6126 TT araC PBSD crap ΔPrrt−6126 TT araC PBSD fur Δprrt−2426 Δlfdgd−6126 ΔlfdgpA198ΔaraC PBSD lac T Δlpar8125 Δgmdfcl81 Δserovar genotype/phenotype ΔaraC lac T Δlpar8125 Δgmdfcl81 Δserovar genotype/phenotype ΔaraC| [13] |
| χ3761       | Typhimurium        | wild type           | [40] |
| χ3744       | Typhi              | ISP1820 wild type   | [41] |
| χ3769       | Typhi              | Ty2 (ρpS)           | [42] |
| χ8438       | Typhi              | Ty2 ρpS+            | [43] |

Plasmid | Descriptionb |
|---------|--------------|
| pWSK129 | pSC101 ori, Kanr |
| pYA3493 | pBR ori, Asd+ vector with bla SS-based periplasmic antigen secretion |
| pYA4088 | Encodes the α-helical region of PsaA (aa 3-285) in phA3493 |

In genotypic descriptions, the subscripted number refers to a composite deletion and insertion of the indicated gene. P, promoter; TT, T4 ip II transcription terminator.

*ori, replication of origin; SS, secretion signal; Kanr, kanamycin resistance.

arabinose and 0.1% mannose at 37°C. Some experiments included KT broth, which is a proprietary medium used to support rapid, high-density bacterial growth, similar in composition to terrific broth [13]. For antibiotic selection of strains containing pWSK129, kanamycin was used at a concentration of 30 µg/ml. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) or Thermo Fisher Scientific (Pittsburgh, PA, USA) unless otherwise indicated.

Formulation stability assays
Strains were grown in KT broth to an optical density at 600 nm of 2.0, then were pelleted and resuspended in phosphate buffered saline (PBS) at 5 x 1010 CFU/ml. Cells were diluted 1:15 into either a 1.3% sodium bicarbonate solution or Ensure (milk chocolate flavor) and incubated at 37°C for four hours. Viability at each time point was assessed by serial dilution and plating onto LB agar containing 0.2% arabinose.

Gastric transit assays
This study was approved by the Arizona State University Institutional Animal Care and Use Committee. Six week old, female BALB/c mice (Charles River Laboratories, Wilmington, MA, USA) were fasted without food or water for 6 h prior to the start of the experiment. Mice received the histamine H1-receptor antagonist chlorpheniramine (0.3 mg/kg) subcutaneously to prevent allergy/anaphylaxis symptoms. Prior to inoculation, low gastric pH was induced by subcutaneous injection of histamine dihydrochloride (10 mg/kg) [37,38]. All bacterial strains used in the gastric transit assays contained the low copy number plasmid pWSK129 (Kanr) to allow for precise quantitation of strain numbers in the non-sterile environment of the gastrointestinal tract. We did not observe any Kanr organisms in the normal intestinal flora of the mice. Strains were grown to late log phase (optical density at 600 nm of 0.9), then pelleted and resuspended in PBS at a concentration of 5 x 1010 CFU/ml. Groups of 5 mice were orally inoculated 50 min after the administration of histamine [29]. For each inoculation, the low gastric pH was treated with sodium bicarbonate, Ensure, or left untreated. Groups that were treated with bicarbonate received 40 µl of a 1.3% sodium bicarbonate solution orally 10 minutes prior to inoculation and an additional 10 µl 10 minutes after [17]. Groups that were treated with Ensure received 20 µl of Ensure (milk chocolate flavor) 10 minutes prior to inoculation and an additional 20 µl 10 minutes after [32]. Mice were euthanized 1 h after inoculation and the entire small intestine was removed, homogenized and serially diluted. Samples were plated onto LB agar containing 0.2% arabinose with kanamycin to determine the number of viable bacteria present following low pH gastric transit.

Statistical analyses
All statistical analyses were performed using GraphPad Prism version 6.00 for Windows (GraphPad Software, La Jolla California USA). Statistical analyses of data from the gastric transit assays were performed using the Mann-Whitney test. Survival curves were analyzed using Sidak’s multiple comparison test.
Phosphate buffered saline; Kan
live oral vaccine. Vaccine.
91.
serovar Typhi
82.
60.
104.
96.

Authors’ contributions
RC, KB and KR conceived and designed the experiments; KB and AG performed the experiments; RC, KB, AG and KR interpreted the data; and KB and KR wrote the manuscript. All authors read and approved the final manuscript.

Authors’ informations
KB has been involved in preparing live attenuated Salmonella vaccine masterseed for use in clinical trials and in developing new technologies and animal models for Salmonella vaccines to enhance safety and immunogenicity. She is currently working in the private sector.

AG is a research associate at Arizona State University.

RC is a professor at The Biodesign Institute and School of Life Sciences at Arizona State University, Tempe, AZ 85287. He has been working in the field of live attenuated Salmonella vaccines for over 25 years and has conducted and/or contributed to a number of human clinical trials.

Acknowledgements
This work was supported by the Bill & Melinda Gates Foundation Grand Challenges in Global Health grant 37863. The authors would like to thank Hayley Cardamone, Elizabeth Griffith and Giorgio Scarpellini for their excellent technical assistance in conducting this study.

Author details
1 The Biodesign Institute, Arizona State University, Tempe, AZ 85287, USA.
2 School of Life Sciences, Arizona State University, Tempe, AZ 85287, USA.
3 Present address - 23andMe, Inc, 1390 Shorebird Way, Mountain View, CA 94043, USA.

Received: 1 December 2014 Accepted: 12 March 2015
Published online: 29 March 2015

References
1. Curtis IS, Nelson PO, Wang P, Gurr E, et al. Effect of recombinant enterotoxin of enterotoxigenic Escherichia coli on ileal permeability. Gastroenterology. 1991;91:261–9.
2. Curtis IS, Nelson PO, Wang P, Gurr E, et al. Effect of recombinant enterotoxin of enterotoxigenic Escherichia coli on ileal permeability. Gastroenterology. 1991;91:261–9.
3. Curtis IS, Nelson PO, Wang P, Gurr E, et al. Effect of recombinant enterotoxin of enterotoxigenic Escherichia coli on ileal permeability. Gastroenterology. 1991;91:261–9.
4. Curtis IS, Nelson PO, Wang P, Gurr E, et al. Effect of recombinant enterotoxin of enterotoxigenic Escherichia coli on ileal permeability. Gastroenterology. 1991;91:261–9.
5. Curtis IS, Nelson PO, Wang P, Gurr E, et al. Effect of recombinant enterotoxin of enterotoxigenic Escherichia coli on ileal permeability. Gastroenterology. 1991;91:261–9.
6. Curtis IS, Nelson PO, Wang P, Gurr E, et al. Effect of recombinant enterotoxin of enterotoxigenic Escherichia coli on ileal permeability. Gastroenterology. 1991;91:261–9.
7. Curtis IS, Nelson PO, Wang P, Gurr E, et al. Effect of recombinant enterotoxin of enterotoxigenic Escherichia coli on ileal permeability. Gastroenterology. 1991;91:261–9.
32. Frey SE, Lottenbach KR, Hill H, Blevins TP, Yu Y, Zhang Y, et al. A Phase I, dose-escalation trial in adults of three recombinant attenuated Salmonella Typhi vaccine vectors producing Streptococcus pneumoniae surface protein antigen PspA. Vaccine. 2013;31:4874–80.
33. Yang J, Dogovski C, Hocking D, Tauschek M, Perugini M, Robins-Browne RM. Bicarbonate-mediated stimulation of RegA, the global virulence regulator from Citrobacter rodentium. J Mol Biol. 2009;394:591–9.
34. Robbe-Sauvé V, Norel F. The rpoS mutant allele of Salmonella typhii Ty2 is identical to that of the live typhoid vaccine Ty21a. FEMS Microbiol Lett. 1999;170:141–3.
35. Foster JW. The acid tolerance response of Salmonella Typhimurium involves transient synthesis of key acid shock proteins. J Bacteriol. 1999;175:1981–7.
36. Bertani G. Studies on lyogensis. I. The mode of phage liberation by lysogenic Escherichia coli. J Bacteriol. 1951;62:293–300.
37. Chew CS, Chen X, Bollag RJ, Isales C, Ding KH, Zhang H. Targeted disruption of the Lasp-1 gene is linked to increases in histamine-stimulated gastric HCl secretion. Am J Physiol Gastrointest Liver Physiol. 2008;295:G37–44.
38. Furutani K, Aihara T, Nakamura E, Tanaka S, Ichikawa A, Ohtsu H, et al. Crucial role of histamine for regulation of gastric acid secretion ascertained by histidine decarboxylase-knockout mice. J Pharmacol Exp Ther. 2003;307:331–8.
39. Li Y, Wang S, Scarpellini G, Gunn B, Xin W, Wanda SY, et al. Evaluation of new generation Salmonella enterica serovar Typhimurium vaccines with regulated delayed attenuation to induce immune responses against PspA. Proc Natl Acad Sci U S A. 2009;106:593–8.
40. Curtis 3rd R, Hassan JO. Nonrecombinant and recombinant avirulent Salmonella vaccines for poultry. Vet Immunol Immunopathol. 1996;54:365–72.
41. Hone DM, Harris AM, Chatfield S, Dougan G, Levine MM. Construction of genetically defined double aro mutants of Salmonella typhi. Vaccine. 1991;9:810–6.
42. Felix A, Pett RM. The pathogenic and immunogenic activities of Salmonella typhi in relation to its antigenic constituents. J Hyg (Lond). 1951;49:92–110.
43. Santander J, Wanda SY, Nickerson CA, Curtiss III R. Role of RpoS in fine-tuning the synthesis of Vi capsular polysaccharide in Salmonella enterica serotype Typhi. Infect Immun. 2007;75:1382–92.
44. Wang RF, Kushner SR. Construction of versatile low-copy-number vectors for cloning, sequencing and gene expression in Escherichia coli. Gene. 1991;100:195–9.
45. Kang HY, Srinivasan J, Curtiss 3rd R. Immune responses to recombinant pneumococcal PspA antigen delivered by live attenuated Salmonella enterica serovar Typhimurium vaccine. Infect Immun. 2002;70:1739–49.
46. Xin W, Wanda SY, Li Y, Wang S, Mo H, Curtiss 3rd R. Analysis of type II secretion of recombinant pneumococcal PspA and PspC in a Salmonella enterica serovar Typhimurium vaccine with regulated delayed antigen synthesis. Infect Immun. 2008;76:3241–54.