Intestinal Colonization of the Infant Mouse Model
By Attenuated and Virulent Vibrio cholerae Strains

BARBARA CEDRE MARRERO, LUIS GUILLERMO GARCIA IMIA,
HILDA MARIA GARCIA SANCHEZ, MILDREY FARIÑAS MEDINA,
ARTURO TALAVERA CORONEL and JUAN FRANCISCO INFANTE BOURZAC

Finlay Institute, Center of Research and Production of Sera and Vaccine, Havana, Cuba

Received for publication March 5, 1997; accepted June 2, 1998 (97/032).

Abstract

Background: Intestinal colonization of humans with virulent Vibrio cholerae stimulates substantial, lasting immunity against reinfection. The purpose of this study was to evaluate the colonizing capability of various Vibrio cholerae strains which are promising candidates to oral vaccine.

Methods: Infant mouse model modification was used. In order to standardize the method, several parameters were tested, such as culture medium and optimal time of incubation and appropriate number of cells to be inoculated. The following were tested: Vibrio cholerae strain 81, 413, and 251A, which were obtained at the Molecular Biology Department of the National Center for Scientific Research, Havana, Cuba. Their virulence cassettes which code for the main virulence factors were deleted.

Results: Good variance coefficient (VC) was obtained in repeated experiments. The colonizing properties of attenuated Vibrio cholerae strains evaluated by this method correlated well with those observed for parental strains.

Conclusions: Genetically attenuated Vibrio cholera strains have the same intestinal colonization level as their parental strains in the infant mouse model; thus, genetic manipulation does not affect genes that encode for the synthesis of colonization factors.


KEY WORDS: Colonization; Vibrio cholerae; Infant mouse model.

Introduction

Small intestine colonization by Vibrio cholerae is one of the most important steps in the pathogenic mechanisms of the cholera disease (1-3). In order to obtain a safe and effective vaccine against cholera, it is essential to achieve the optimal expression of the factors that ensure adherence and multiplication of bacteria in the small intestine, thus stimulating a protective and long-lasting immune response.

Vibrio cholerae El Tor and Classic biotype produce two colonization factors, MSHA and TCP (4). The inactivation of these protein genes reduces the cell colonization capacity in the “infant mouse model.”

Different studies have demonstrated that bacterial colonization is the most important aspect in the immunogenicity of the microorganism (5,6). On the other hand, bacterial virulence is closely related with its ability to adhere and multiply in the small intestine (4).

The infant mouse model was studied in different conditions in order to evaluate the colonization capabil-
ity of some genetically attenuated *Vibrio cholerae* strains.

The purpose of this study was to evaluate the colonizing capability of various *Vibrio cholerae* strains which are strong candidates to oral vaccine. This paper describes the methodology used and the results of studies on colonization of genetically attenuated strains of *Vibrio cholerae* with reference to their parental virulent. The results of this study have shown that these strains maintain the colonization capacity and the possibility to elicit an antibacterial immune response despite genetic manipulations.

**Materials and Methods**

**Animals.** Intestinal colonization assay was done in inbred Balb/c 2- to 4-day-old mice, with weights ranging from 1.5 - 2 g.

**Bacteria.** The sources and characteristics of the *Vibrio cholerae* strains used in this study are shown in Table 1. Organisms were maintained at -70°C in 10% skim milk containing 20% glycerol.

**Media.** In the first experiment, C6706 and 413 strains were grown in Tryptone Soya Broth (TSB) (OXOID) and in Colonization Factors Broth (CFB) in order to select the best media for the subsequent assays. Cultures were incubated at 37°C, 200 rpm, for 6 h.

**Inoculum Size.** The inoculum was administered by oral route in cellular suspensions with $10^3$, $10^4$ and $10^5$ bacteria in 50 μL of phosphate buffered saline (PBS) pH 7.4 containing 0.01% Evans Blue dye using an eye needle and a syringe. Six animals per dose were used.

**Optimal Incubation Time.** After inoculation with $10^5$ cells, the animals were divided into groups and were sacrificed at 6, 12, 18 and 24 h.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Description</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>C7258*</td>
<td>Wild type, El Tor, Ogawa, Peru 1991</td>
<td>7</td>
</tr>
<tr>
<td>C6706*</td>
<td>Wild type, El Tor, Inaba, Peru 1991</td>
<td>7</td>
</tr>
<tr>
<td>81</td>
<td>ctxA ctxB zot ace orfU cepA</td>
<td>8</td>
</tr>
<tr>
<td>413</td>
<td>ctxA ctxB zot ace orfU cepA</td>
<td>8</td>
</tr>
<tr>
<td>SG 251*</td>
<td>Wild type, O139 serogroup.</td>
<td>9</td>
</tr>
<tr>
<td>251A</td>
<td>ctxA ctxB zot ace orfU cepA</td>
<td>8</td>
</tr>
<tr>
<td>569B*</td>
<td>Wild type O1, Classical, Inaba</td>
<td>10</td>
</tr>
</tbody>
</table>

* Provided by Dr. Richard A. Finkelstein, Department of Molecular Microbiology and Immunology, School of Medicine, University of Missouri, Columbia, MO, USA.

In all cases, groups of six mice were used to test each one of the parameters, and the experiments were repeated three times.

**Colonization Assay.** The method is a modification reported by others (11,12). This assay was made using the best parameters as determined in the experiments described above. Mice were separated from their parents the day before, in order for them to have their stomach empty at the time of challenge. Fifty microliters of cellular suspensions were given by oral route with an eye needle and a syringe. After the incubation time, the animals were sacrificed, the small intestines removed and weighed in groups of ten, and then washed. The intestines were homogenized in PBS at 13,500 rpm for 15 sec in an Ultraturrax T 25 IKA (Labortechnik, Berne, Switzerland).

**Determination of Bacteria Counts.** The colonizing properties of *Vibrio cholerae* were assayed by counting viable vibrios in the intestinal wall after it had been washed. Numbers of bacteria recoverable from mice were determined by homogenization in PBS of pooled intestines from ten mice in each group. Suitable dilutions in PBS of the homogenates were plated on Thiosulphate-Citrate-Bile salt-Sucrose (TCBS) (OXOID) agar for viable counting, and were cultivated for 24 h. The results were expressed as CFU/g of intestine.

Analysis of variance was used for comparing the index of colonizing of attenuated and virulent strains.

**Results**

*Vibrio cholerae* C6706 and 413 strains were chosen in order to select the best culture medium, the former because it is a wild-type strain, and the latter because it is a manipulated strain, comparing their behavior in both media. Figure 1 shows the comparison between TSB and
VIBRIO CHOLERA COLONIZATION IN INFANT MOUSE

VIBRIO CHOLERA COLONIZATION IN INFANT MOUSE

Figure 2. Evaluation of different inoculum sizes in the infant mouse model. Cells were grown in TSB medium.

CFB media. There is no difference in CFU/g of intestine, regardless of the media used to culture the cells. On the other hand, the results were very similar for both strains.

The experiment to select the inoculum size is summarized in Figure 2. The experiment was repeated three times and, in all cases, the highest colonization index was obtained with 10^5 CFU for the strains used. In this case, two strains were chosen, a highly virulent, C7258, and a strain with a low colonization index, 569B strain.

Higher doses were not assayed because they constitute the 50% lethal dose (LD_50) for both of these strains (data not shown). Figure 3 presents the results for three different strains and four incubation times. For all strains, the 24-h period from inoculation to animal sacrifice showed the highest values of CFU/g of intestine. Other periods of time were not proved, because at that time, all strains were capable of colonizing small intestine.

The results of the above studies have allowed us to determine the best parameters and design the experiment to compare the colonization index of different VIBRIO CHOLERA strains (Figure 4). The strains tested showed similar colonization indexes, except VIBRIO CHOLERA

Figure 3. Kinetics of colonization in the infant mouse model of V. cholerae strains. Animals were sacrificed at different times after inoculation of 10^5 cells.

Figure 4. Colonization index of different V. cholerae strains in infant mouse model. Groups of 10 animals were inoculated by oral route with viable V. cholerae strains and sacrificed 24 h later.

Classical Inaba 569B strain, with low variation coefficient. Variation coefficients (VC) expressed in percent (%) of the studied strains in this animal model in this study were from 1.43 - 6.63 for transformed strains and 2.42 - 8.04 in the epidemic strains.

Discussion

VIBRIO CHOLERA is a non-invasive pathogen, and consequently, adherence and colonization are critical factors in the disease process. The infant mouse model has been widely used in the evaluation of the virulence and colonization capacity of VIBRIO CHOLERA (11-13).

MSHA is one of the colonization factors in VIBRIO CHOLERA El Tor strains, and other authors have obtained the best expression levels of this protein using TSB as a culture medium (14). In addition to MSHA, other antigens and colonization factors are expressed in CFB (15).

TSB and CFB were used to grow the vibrios. Vibrios cultured in both media showed the same adhesive properties. The TSB medium was selected because it is easier to prepare and has a high yield in biomass production.

To determine the optimal inoculum size, 10^3, 10^4 and 10^5 CFU of the well-known VIBRIO CHOLERA strains C7258 and 569B were inoculated. The former is a very virulent strain isolated from a cholera patient in Peru, and several reports have been done on the poor colonization capability of the 569B strain. The latter shows diminished intestinal colonization in humans (16), mice (17) and rabbits (18). In 24 h, both strains had colonized the small intestine of infant mice but with significant differences (p <0.025).

VIBRIO CHOLERA 81 and 569B strains were unable to colonize the intestine both at 6 and 12 h after inoculation, the last one even at 18 h. Only 24 h after the inoculum administration, all strains achieved measurable levels of intestinal colonization.
Vibrio cholerae C7258 and C6706 strains, isolated from cholera patients in Peru, as well as SG 251, increased their cellular concentration in the intestine, 10^6 and 10^7 times with respect to the initial inoculum. Similar results were obtained with attenuated strains 81, 413, 251A, produced in the Molecular Biology Department of the National Center for Scientific Research (8). These strains have proven to be avirulent in the Rabbit Ileal Loop model, and have LD50 values between 10^2 and 10^3 higher than the virulent parental strains (8). On the other hand, the 569B strain showed a very low colonization level, with an increase of only 100 times the cell number per gram of intestine.

Analysis of variance indicates the non-existence of significant differences between parental epidemic strains and their transformer strains.

Even though references concerning the precision of the model used do not appear in the literature in general, calculated variation coefficients are lower than those accepted, even for immunoenzymatic assays, which are reported to be lower than 10 - 11% (19,20).

The fact that the parental strains and the attenuated strains present similar colonization indexes is in agreement with the value expected, because in the genetic transformation, the genes that regulate the adherence capability were not altered in the study.

The results obtained concerning the VC indicate that this model presents an acceptable precision, and is able to distinguish among strains of different colonizing capabilities.

The principal conclusion of this work is that under the experimental conditions used here, genetically attenuated Vibrio cholerae strains have the same intestinal colonization level as their parental strains in the infant mouse model. It seems that genetic manipulation does not affect genes that encode for the synthesis of colonization factors.

Acknowledgments

The authors wish to express appreciation to Jorge Benitez for providing attenuated strains, and to Dr. Gustavo Sierra for critical review of the results of this work.

References