Immunological Conversion of Vibrio cholerae in Gnotobiotic Mice

C. E. MILLER, K. H. WONG, J. C. FEELEY, AND M. E. FORLINES

Laboratory of Bacterial Products, Division of Biologics Standards, National Institutes of Health, Bethesda, Maryland 20014

Received for publication 4 May 1972

Cholera vibrios grow readily in the intestines of gnotobiotic mice and change their antigenic structure in response to immunological pressures. These changes were progressive, with the rough form persisting as the apparent end point in the mice. Inoculation of the rough form into fresh gnotobiotic mice resulted in the recovery of smooth forms. An untypable rough strain of Vibrio cholerae isolated from a chronic carrier in Calcutta in 1967 was passed serially in gnotobiotic mice. Smooth strains of V. cholerae were recovered from the feces after selective treatment with complement and antiserum against a rough strain. They were confirmed as O group I cholera vibrios belonging to the eltor biotype. The ability of these strains to produce diarrheal fluid in infant rabbits and to increase capillary permeability in rabbit skin was markedly increased over that of the original human isolate. This demonstrates that rough avirulent forms of V. cholerae from human carriers can undergo antigenic changes and concurrently increase in virulence when placed in an appropriate environment. The many inapparent cholera infections in endemic areas may provide a mechanism by which V. cholerae changes its serotype away from the immunological pressures in the population.

The ability of Vibrio cholerae to adjust to immunological pressure in vivo has been demonstrated in gnotobiotic mice (18). Inoculation of either an Ogawa or Inaba strain into these bacteria-free mice resulted in a persistent asymptomatic infection. Within 2 weeks after exposure, colonies of the opposite serotype began to appear in the stool and increased in number, only to be replaced by rough vibrios in a few weeks. This disappearance of smooth strains of V. cholerae was correlated with the appearance of circulating antibody in these mice. The rough forms of V. cholerae persisted for many weeks and appeared to represent the end stage of change in any given group of mice. However, propagation of the progeny of one of these rough colonies in fresh gnotobiotic mice produced smooth cholera vibrios not unlike the original culture.

Chronic carriers of V. cholerae have been identified in areas of endemic disease. Three carriers were identified among 81 patients hospitalized in Calcutta, India, during the 1967 cholera season (15). All three patients had Ogawa, biotype eltor, infections on admission. One patient shed only rough vibrios during the 331 days of study before treatment with antibiotics. Immunization of a rabbit with these organisms produced detectable vibriocidal antibody levels in the blood, indicating the presence of some smooth O antigens in these rough organisms. Pierce (15) suggested that these forms might be thought of as "partially rough," and capable of reverting to the smooth form in an appropriate environment. The gnotobiotic bacteria-free mouse seems to provide such an environment. Our findings with this rough culture in gnotobiotic mice are presented.

MATERIALS AND METHODS

Cultures. The rough culture of V. cholerae was supplied by N. F. Pierce on a nutrient agar slant. Subsequent growths for inoculation and colony counts were on Trypticase gelatin agar (1% Trypticase [BBL], 1% NaCl, 3% gelatin, 1.5% agar, pH 7.4). The smooth culture of V. cholerae strain VC-12 was classical Ogawa isolated in Dacca, East Pakistan, and stored at 4 C in the lyophilized state (11).

Mice. The mice were the National Institutes of Health strain of gnotobiotic, bacteria-free mice maintained in germ-free isolators. The absence of bacteria was determined as previously described (18).

Inoculation and culture procedures. These mice were inoculated by placing 0.1 ml of culture suspen-
sion in the esophagus through an intradermic poly-
ethylene tube (PE 20) connected to a 25-gauge
needle. Ten mice were used in each experiment. A
composite sample of feces from all 10 mice was ob-
tained for a weekly stool culture. Five milliliters of
phosphate-buffered saline, pH 7.4, containing 0.1%
gelatin was added to emulsify the feces. The solid
material was removed by centrifugation at 700 rev/
min for 15 min. The supernatant material was used in
the following procedures.

Selection of smooth forms. One milliliter of the
fetal extract was placed in a tube with 2 ml of 1:5
antirough serum ("X") antisera supplied by H. L.
Smith, Jr., Jefferson Medical College, Philadelphia,
Pa.) and 1 ml of guinea pig complement (lot 3-4837,
Microbiological Associates, Inc., Bethesda, Md.)
(16). This mixture was incubated in a 37°C water
bath for 4 hr. A mixture of 1 ml of fecal extract and
3 ml of gelatin-buffered saline was incubated as a
control. After incubation, serial 10-fold dilutions were
made of each mixture, and 0.2 ml was spread on each
of two Trypticase gelatin agar plates. The plates were
incubated overnight at 35°C. Colony counts were
recorded, and isolated colonies from the plates of the
treated fecal extract were typed by slide agglutination
with Ogawa, Inaba, and antirough sera and 1:1,000
acriflavine. A colony was considered rough if it ag-
glutinated either in antirough serum or acriflavine.
One promising candidate for smooth form from the
second weekly fecal sample was selected on the basis
of its slide-agglutination pattern. This culture was
checked for stability by serial transfers on Trypticase
gelatin agar plates for 10 days. It was then inoculated
into a new group of 10 mice. Selection of the culture
for the third animal passage was made from the feces
of these mice. Several cultures isolated from the third
animal passage were saved on Trypticase gelatin agar
slants for study in comparison with the original rough
culture isolated from a human carrier. Efforts were
made to include a representative of each agglutination
pattern. The Heiberg grouping (8) of each isolate was
determined by rapid sugar fermentation (12) in
succrose, mannose, and arabinose. Each isolate was
also tested for chick cell agglutination (4) and re-
sistance to polymyxin B (6).

Assay for fluid production and capillary permeability
factor. Two tests were employed to determine the
relative enterotoxin-producing capacity of the new
isolates and the original rough culture of V. cholerae.
The VC-12 culture was included as a smooth positive
control. First was the comparison of whole cultures
in 9-day-old infant rabbits (5). Each culture was
grown overnight at 30°C on a shaker in a 500-ml
flask with 50 ml of complete Syncase medium con-
taining 0.1% sucrose (17). The infant rabbits were
fasted overnight and given 5 ml of culture via stomach
tube (PE 50 on a 23-gauge needle). The rabbits were
fed 5 ml of infant formula (Similac) twice daily by
stomach tube. All dead animals were necropsied to
determine the cause of death. Only those animals with
the intestines distended with rice water were con-
sidered to have died from cholera. The experiment
was terminated at the end of 48 hr.

The second test was the assay of capillary permea-
bility activity of a sterile culture filtrate in the skin of
young adult rabbits (2). The cultures were grown in
complete Syncase media with 0.1% sucrose at 30°C,
as above. Each culture was centrifuged at 9,000 rev/
min at 4°C. The supernatant fluid was filtered through
a 1-μm membrane filter (Millipore Corp., Bedford,
Mass.), and then a 0.20-μm membrane filtration unit
(Nalgene Labware, Fisher Scientific Co.). The first 15
to 20 ml of filtrate was discarded, and the remainder
was saved for skin tests. Dilutions were made 1:5
and 1:25 with phosphate-buffered saline, pH 7.4, con-
taining 0.1% gelatin. Intradermal injections were
given with 0.1 ml of material in duplicate randomized
sites on two rabbits. At the end of 24 hr, each rabbit
was injected intravenously with 5 ml of 1% Evans
blue dye as an indication of increased capillary per-
meability at the injection site. Readings were taken
after 1 and 18 hr.

RESULTS

Selection of smooth V. cholerae. The total vibrio
count from the extracts of mouse feces was essen-
tially constant in all the mice (Fig. 1). Treatment of the
cecal extract with guinea pig complement and antirough
serum effectively reduced the number of rough colonies and thus
facilitated isolation of smooth forms. However, these surviving colonies in the first and second mouse passage
were not completely smooth. While they lost the ability to agglutinate in antirough serum, they all retained the ability
to agglutinate in acriflavine.

It was not until the third mouse passage that completely smooth colonies began to appear (Fig. 2). The number of smooth isolates in-
creased to 79% by the second week, before being replaced by rough forms in later weeks. The Ogawa, Inaba, and Hikojima serotypes and
the two rough cultures were obtained from these mice. The cultures were saved on gelatin agar slants for the following studies.

Biochemical tests. All five derived cultures (i)
agglutinated in group I antiserum, fermented
succrose, and mannose, but not arabinose, (ii)
were resistant to polymyxin B, and (iii) ag-
glutinated chick cells (Table 1). Based on poly-
myxin sensitivity and chick cell agglutination,
they were all considered eltor cholera vibrios, the same as recovered from the convalescent carrier.

Relative virulence. The ability of these cultures to produce cholera-like syndrome and death in infant rabbits is shown in Table 2. The Inaba isolate was nearly as virulent as the VC-12 positive control, killing five of six rabbits in 48 hr. The other four isolates contributed 6 of the total of 11 deaths in 48 hr, whereas the original culture produced no mortality. All positive controls, but none of the negative controls, died of cholera.

Bluing reactions caused by culture filtrates of these organisms in the rabbit skin test are shown in Table 2. Each isolate from the mice produced a bluing reaction much larger than the original culture. The 1:5 dilution of the Ogawa isolate produced a bluing area similar to the undiluted material from the original culture. One of the rough isolates from gnotobiotic mice had some activity in the 1:5 dilution. None of the isolates had any activity in the 1:25 dilution, whereas the VC-12 culture produced a reaction.

**DISCUSSION**

This study supports the suggestion of Pierce et al. (15) that rough *V. cholerae* from convalescent carriers, if placed in an appropriate environment, could become typical smooth *V. cholerae*. In gnotobiotic mice, the changes were

**TABLE 1. Serological and biochemical reactions of Vibrio cholerae isolates from mice**

<table>
<thead>
<tr>
<th>Culture</th>
<th>Slide agglutinations</th>
<th>Polymyxin B sensitivity</th>
<th>Chick cell agglutination</th>
<th>Carbohydrate fermentation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Antisera</td>
<td>Ac</td>
<td></td>
<td>Suc</td>
</tr>
<tr>
<td></td>
<td>Gp I</td>
<td>Og</td>
<td>In</td>
<td>X</td>
</tr>
<tr>
<td>Original</td>
<td>+</td>
<td>ND</td>
<td>ND</td>
<td>+</td>
</tr>
<tr>
<td>Ogawa b</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Inaba b</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Hikojima b</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Rough O b</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Rough X b</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>VC-12</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

a Abbreviations: Gp I, Heiberg group I; Og, Ogawa; In, Inaba; X, antirough; Ac, 1:1,000 acriflavine; Suc, sucrose; Man, mannose; Arab, arabinose; ND, not done.
b Isolates from third mouse passage of rough *V. cholerae*.

**TABLE 2. Biological activities of Vibrio cholerae isolates in rabbits**

<table>
<thead>
<tr>
<th>Culture</th>
<th>Infant mortality (48 hr)</th>
<th>Bluing in skin (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Undiluted</td>
<td>1:5</td>
</tr>
<tr>
<td>Original culture</td>
<td>0/6</td>
<td>3.5</td>
</tr>
<tr>
<td>Ogawa b</td>
<td>1/6</td>
<td>9.6</td>
</tr>
<tr>
<td>Inaba b</td>
<td>5/6</td>
<td>5.6</td>
</tr>
<tr>
<td>Hikojima b</td>
<td>2/6</td>
<td>9.1</td>
</tr>
<tr>
<td>Rough O b</td>
<td>2/6</td>
<td>6.5</td>
</tr>
<tr>
<td>Rough X b</td>
<td>1/6</td>
<td>10.8</td>
</tr>
<tr>
<td>Negative control</td>
<td>0/6</td>
<td>0</td>
</tr>
<tr>
<td>Positive control</td>
<td>6/6</td>
<td>13.3</td>
</tr>
</tbody>
</table>

a Mean of four sites on two rabbits.
b Isolates from third mouse passage of rough *V. cholerae*. 

**FIG. 2.** Percent smooth colonies of *V. cholerae* in isolates from feces of gnotobiotic mice inoculated with rough *V. cholerae* (third passage in bacteria-free mice). Number at top of bar indicates number of colonies tested in sample.
gradual and progressive. Three serial transfers into unexposed mice were necessary before completely smooth colonies were obtained. Nearly 21% of the colonies tested were able to agglutinate acriflavine, and prolonged exposure in the same mice only produced more rough colonies. *V. cholerae* seems to be very adaptable to environmental pressures. Early workers (9, 14) defined three cholera serotypes: Ogawa, Inaba, and Hikojima. These serotypes share group-specific somatic antigens but differ in minor type-specific O antigens. Ogawa and Inaba are usually designated by the antigenic symbols AB and AC, respectively, while Hikojima shares all three antigens ABC. The change from B to C antigen has been observed in natural infections (7), and demonstrated in the laboratory in vitro (1, 19) as well as in vivo (18). The serotypical changes in cultures from gnotobiotic mice were progressive with time, leading to the eventual loss of the smooth strains and the appearance of the rough forms. Rough colonies have also been observed at the end of the natural disease in man as well as in chronic carriers (3).

*V. cholerae* in the rough form can exist for many months in man (15) or gnotobiotic mice (18) with no evidence of disease. Pierce monitored one carrier of rough vibrios for 11 months before the condition was terminated by treatment with antibiotics. The antibody response of this convalescent carrier was considered normal by the usual tests (15). The peak titers occurred between days 8 and 16, and then declined steadily despite the persistence of rough *V. cholerae* in the intestines.

*V. cholerae* is seasonal in appearance in endemic areas. In a 2.5-year study of a rural area in East Pakistan, McCormack et al. (10) reported periods of 2 to 5 months when no infections were detected. Over 21,000 rectal swabs were cultured from persons reporting diarrhea during these cholera-free months. In addition, *V. cholerae* was not recovered from the local water tanks during these periods. Yet when cholera reappeared in the area under study, it was found in many villages almost simultaneously.

The source of the first cases in a cholera season usually remains a mystery. Carriers may provide a link between epidemic seasons when *V. cholerae* cannot be found in the environment. When clinical cholera did appear, many inapparent infections were detected. These occurred mainly in persons with low antibody titers (13). These inapparent infections could serve as a mechanism for *V. cholerae* to change its serotype away from type-specific antibody present in the community. The lack of type-specific antibody in one patient appeared to allow a relapse due to Inaba infection 10 days after the patient was hospitalized with an Ogawa infection (7). Results of our studies in gnotobiotic mice are consistent with these contentions.

**ACKNOWLEDGMENTS**

We wish to thank N. F. Pierce, Baltimore City Hospital, Baltimore, Md., for the culture from the convalescent carrier.

**LITERATURE CITED**


