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# Attenuation of virulence by P and V plasmids in *Vibrio cholerae*: strains suitable for oral immunization *

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*In a virulent strain of* Vibrio cholerae, *KB9, P and V plasmids were introduced by bacterial conjugation. Characterization of PV isolates and systematic screening of them in animal models of cholera revealed that a large number of PV isolates were non-pathogenic, owing to the loss of ability to synthesize toxin. Results obtained with two such strains, designated as KB9:PV and CD24, are described. The strains with plasmids were stable during* in vitro *cultivation or during two successive passages in rabbit intestine. Protection conferred by PV strains was determined in mouse protection tests and in the rabbit ileal loop model. The plasmid strains were immunogenic. In view of the results, it is proposed that PV-bearing attenuated strains should be tried in oral immunization.*

Several attempts have been made to isolate attenuated strains of *Vibrio cholerae* suitable for use as live oral vaccines (for reviews see *7, 13*). Unfortunately, some of these strains have proved to be unsatisfactory (*3*).

In an attempt to isolate new promising strains, we discovered that the pathogenicity of *V. cholerae* was significantly suppressed by introducing the P and V plasmids (*12*). The present paper demonstrates a new procedure for producing attenuated PV strains of *V. cholerae*. Plasmids did not alter the immunogenicity, and they were not lost *in vitro* or *in vivo*.

## MATERIALS AND METHODS

### *Vibrio strains*

The *V. cholerae* strains used, with their relevant markers and plasmid status, are shown in Table 1. Details of the maintenance and isolation of strains harbouring plasmids have been described elsewhere (*12*).

### *Culture media*

Nutrient broth (Difco) and brain heart infusion (BHI) (Difco) were prepared according to the manufacturer's directions. "Syncase" sucrose minimal medium was prepared as described by Finkelstein & Lospalluto (*8*). Phosphate-buffered saline (PBS) and minimal medium supplemented with the required amino acids were prepared as described earlier (*12, 2*). All the media, when required, were solidified with 1% agar powder (Oxoid) and were autoclaved at 103 kPa (15 lbf/in²) for 10 min.

Table 1. *Vibrio cholerae* strains used in these experiments

| Strain | Relevant markers | | Plasmid status | Reference or source |
|---|---|---|---|---|
| KB9 | *ilv arg his, str*$^r$, | O-Og | P-V- | *2* |
| KB9:P | *ilv arg his, str*$^r$, | O-Og | P+V- | *2* |
| KB9:V | *ilv arg his, str*$^r$, | O-Og | P-V+ | *2* |
| KB9:PV | *ilv arg his, str*$^r$, | O-Og | P+V+ | *2* |
| KB92 | *Prototroph,* | O-Og | P-V- | K. Bhaskaran |
| KB92:P | *Prototroph,* | O-Og | P+V- | *12* |
| KB93 | *Prototroph,* | O-In | P-V- | K. Bhaskaran |
| CD24 | *ilv, arg his, str*$^r$, | O-Og | P-V+ | This work |
| 569B | *Prototroph,* | O-In | P-V- | K. Bhaskaran |
| 569B:P | *Prototroph,* | O-In | P+V- | *12* |
| 569B:V | *Prototroph,* | O-In | P-V+ | *12* |
| CD23 | *Prototroph,* | O-In | P+V+ | This work |

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*BHI culture*

Broth culture and growth conditions have been reported earlier (*12*). Bacterial dilutions were made in PBS.

*Animal experiments*

Mouse virulence tests and ileal loop experiments in adult rabbits (*4*) were performed as described elsewhere (*12*).

The 10-day-old infant rabbit model (*5*) was employed for the enteropathogenicity test. The animals were infected intraintestinally with 1 ml of culture suspension and the occurrence of diarrhoea or death was noted up to 48 h. Later, an autopsy was done and the amount of fluid accumulated in the caecum was recorded.

The mouse protection test was based on the method of Pittman & Feeley (*11*). Swiss mice, 10–12 g in weight and reared in this Institute, were immunized by injecting intraperitoneally $3–7.4 \times 10^6$ organisms. These immunized mice were challenged intraperitoneally on day 15 with 500 $LD_{50}$ of homologous (KB92, Ogawa) and heterologous (KB93, Inaba) virulent strains. The challenge dose was prepared in 5% mucin (Type 1701-W, Wilson Laboratories, Ill.). Mortality was recorded up to 72 h.

The ligated ileal loop model in the adult rabbit (*4*) was employed for determining the immunogenicity of the attenuated strains. Two doses of live vaccine (BHI culture), each of 1 ml, were administered subcutaneously with an interval of 21 days. The vaccine was prepared on the day of immunization. The bacterial counts varied from 1.24 to $1.86 \times 10^9$ per ml. All the rabbits were bled before immunization and before the challenge. Two ileal loops were made in each rabbit for challenge with homologous (KB92, Ogawa) and heterologous (KB93, Inaba) strains. Challenge inoculum consisted of approximately $10^6$ organisms contained in 1 ml. A postmortem was performed after 18 h. Loop contents and length were measured in order to determine the immunity to challenge. Serum vibriocidal antibodies were measured by Finkelstein's method (*6*).

*Preparation and assay of cholera toxin*

The preparation of the toxin and its assay have been described earlier (*12*).

*Preparation of strains with P and V plasmids*

This, together with detection of P and V plasmids, have been described in earlier papers (*2, 12*).

## RESULTS

*Attenuation of virulence*

*Mouse virulence test.* The $LD_{50}$s of the virulent parent strain, KB9, and its attenuated derivatives, KB9:PV and CD24, were determined. The $LD_{50}$ of KB9 was approximately 9 organisms, whereas the $LD_{50}$s of KB9:PV and CD24 were found to be $2.77 \times 10^6$ and $4.2 \times 10^7$ organisms, respectively.

*Enteropathogenicity in rabbits.* In the adult rabbit model, $1.2–1.6 \times 10^9$ organisms of KB9:PV and CD24 were administered to each ligated ileal loop but they failed to cause any outpouring of fluid. In similar tests, $1–1.3 \times 10^4$ organisms of KB9 produced a significant amount of fluid (*12*).

The nonpathogenic nature of plasmid-carrying strains was further confirmed by experiments with the infant rabbit model. None of the 16 infant rabbits infected with KB9:PV or CD24 showed any evidence of diarrhoea or accumulation of fluid in the caecum. Postmortem findings revealed a normal appearance of the gut. On the other hand, rabbits infected with KB9, with a dose as low as $1.4 \times 10^5$ organisms, developed diarrhoea, and the postmortem examination done after 48 h showed a characteristic distension of the caecum.

*Assay of toxin in guinea-pigs.* Earlier we reported that a strain of 569B harbouring both P and V plasmids produced less toxin *in vitro* (*12*). Since previously pathogenic strains lost their pathogenicity completely when carrying P and V, several strains of 569B containing P and V were prepared and their capacity to synthesize toxin *in vitro* was examined. Most of the strains harbouring P and V did not produce any detectable amount of toxin, whereas under identical conditions 569B gave a significant quantity of toxin. A bacteria-free culture filtrate of CD24 did not produce induration in the skin of the guinea-pigs.

*Stability of plasmids*

Our present findings show that the inclusion of P and V plasmids in *V. cholerae* strains causes attenuation of virulence in various experimental models. Therefore, it becomes necessary to examine the stability of these plasmids.

*In conjugation.* A conjugation experiment between KB9:P and KB92 showed that while the P plasmid was transferred to KB92 with normal frequency, KB9:P did not become $P^-$. This was revealed in the following experiment. The two strains were plated on a streptomycin nutrient agar, which allowed only the donor to grow. Simultaneously,

samples were plated on minimal medium on which only KB92 would grow. It was found that 100% of the donor cells were $P^+$ whereas 44–64% of KB92 were $P^+$. Hence, the donor cells always retained a copy of the P plasmid. This is suggestive of plasmid replication during sex transfer.

*In vivo.* Strains KB9:P and KB9:V were given two serial passages in the ileal loop of adult rabbits. About 100 colonies, isolated after animal passage, were tested for their plasmid status. In no instance was there any loss of the plasmids.

*In vitro.* Incubation with a nonlethal concentration of acridine orange and sodium dodecyl sulfate did not eliminate the plasmids from cells.

Two successive subcultures of plasmid-bearing strains in BHI did not eliminate plasmids. Strains KB9:P, KB9:V, and KB9:PV were kept for 3–4 weeks at 37°C without agitation. Discrete colonies were obtained on nutrient agar plates. In all, 144 colonies were tested and all of them were found to have retained their respective plasmids. When KB9:PV was incubated for 4 weeks, 7 colonies did not show the P plasmid. CD24 was one of these isolates. On the assumption that CD24 harboured only V, this strain was crossed with a $P^+$ strain to transfer P into CD24. In two independent matings, P could not be transferred to CD24. A control was also run with KB9:V in which P transfer occurred with normal frequency. This suggests that CD24 is not indeed devoid of P, which may be present in a repressed state (*2*).

### *Immunogenicity of KB9:PV and CD24*

Before using them as vaccines, it is necessary to determine whether the attenuated strains are able to confer immunity.

*Mouse protection test.* Table 2 demonstrates the protection conferred by these strains in the mouse protection test. It appears that all the strains are quite immunogenic and give rise to good protection, particularly against homologous (KB92, Ogawa) challenge.

Table 2. Protection conferred by attenuated strains in the mouse protection test[a]

| Immunizing strain | No. of organisms in immunizing dose | No. of deaths/no. challenged after challenge with: | |
|---|---|---|---|
| | | KB92 | KB93 |
| KB9 | $4.0\times10^6$ | 0/16 | 3/16 |
| KB9:P | $3.0\times10^6$ | 0/16 | 7/16 |
| KB9:V | $6.1\times10^6$ | 0/16 | 5/16 |
| KB9:PV | $7.4\times10^6$ | 1/16 | 10/16 |
| CD24 | $5.5\times10^6$ | 1/16 | 6/16 |

[a] Challenge dose was 500 $LD_{50}$.

*Ileal loop challenge experiments in adult rabbits.* The degree of protection conferred by live bacterial vaccines prepared from attenuated strains KB9:PV and CD24 was investigated in adult rabbits. The immunogenic efficacy of the two strains was evaluated against homologous (KB92, Ogawa) and heterologous (KB93, Inaba) challenge strains. The immunized rabbits were challenged 35 days after the second dose of vaccine. Immunity to challenge and assay of vibriocidal antibodies was determined.

Table 3 shows that both the vaccines induced significant protection against the two challenge strains, within the range from 73 to 100%. High vibriocidal titres were also seen towards the chal-

Table 3. Ileal loop challenge experiments in adult rabbits at 35 days after the second dose of vaccine

| Vaccine strain | No. of rabbits | Challenge strain | Fluid in ileal loop[a] | | Immunity to challenge (%) | Vibriocidal titre[b] (mean) |
|---|---|---|---|---|---|---|
| | | | Range | Mean | | |
| KB9:PV | 7 | KB92 | 0.00-1.58 | 0.37 | 73 | $2.23\times10^5$ |
| | | KB93 | 0.00-0.00 | 0.00 | 100 | $8.71\times10^4$ |
| CD24 | 6 | KB92 | 0.00-1.65 | 0.27 | 80 | $1.00\times10^5$ |
| | | KB93 | 0.00-1.33 | 0.39 | 76 | $7.78\times10^4$ |
| Control | 7 | KB92 | 0.00-2.13 | 1.39 | — | <50 |
| | | KB93 | 1.23-1.88 | 1.65 | — | <50 |

[a] ml/cm of loop.

[b] Titre is the reciprocal of the highest dilution causing a positive reaction. The vibriocidal titre of the pre-immunized sera was less than 50.

Table 4. Multiplication and adherence of vibrios in the ileal loop of adult rabbits

| Strains | No. of vibrios injected | No. of vibrios adhering to wall of intestine (per ml) | No. of vibrios in lumen (per ml) | Total no. of vibrios (per ml) | Fluid in ileal loop (ml/cm) |
|---|---|---|---|---|---|
| KB9 | $1.4 \times 10^5$ | $6.0 \times 10^8$ | $7.5 \times 10^9$ | $8.1 \times 10^9$ | 1.63 |
| KB9: PV | $3.6 \times 10^4$ | $2.3 \times 10^8$ | $2.1 \times 10^9$ | $2.33 \times 10^9$ | 0.00 |
| CD24 | $1.2 \times 10^5$ | $7.5 \times 10^8$ | $7.5 \times 10^9$ | $8.3 \times 10^9$ | 0.00 |

lenge strains to which the animals were immune. Findings are suggestive of good protection associated with a high titre of vibriocidal antibodies.

*Multiplication and adherence of KB9: PV and CD24*

The growth of the plasmid-carrying strains was compared with that of the parent KB9 and no difference was found *in vivo* or *in vitro*. Adherence of CD24 and KB9: PV to the intestine was similar to that of KB9. The data recorded in Table 4 suggest that bacterial adherence to the intestinal mucosa and multiplication in the lumen were not affected.

## DISCUSSION

Two strains of *V. cholerae* harbouring P and V plasmids demonstrated loss of pathogenicity in various experimental models. In an earlier report we described the suppression of pathogenicity by these plasmids (*12*). The present study was more extensive, using a large number of recombinants harbouring both these plasmids, and we investigated whether infection of *V. cholerae* with these plasmids caused loss of pathogenicity due to loss of ability to produce biologically active toxin. It was also observed that these attenuated strains do have the ability to multiply and adhere in the rabbit gut, which is a vital point for a strain that may be used for the production of a live oral vaccine (*7, 9*).

The plasmid status of these strains appears to be quite stable both *in vitro* and *in vivo*. Some of these strains have been maintained and passaged on nutrient agar for several years and loss of plasmid(s) has not been observed so far. Successive passages in rabbit intestine do not have any effect on the plasmids. It has been reported that plasmid P can be eliminated by lengthy incubation on solid medium (*1*) and by sodium dodecyl sulfate (*10*). However, none of these treatments, including exposure to acridine orange, eliminated these plasmids in our studies. Lengthy incubation in BHI had no effect, except in one instance where P appears to have been lost, resulting in the isolation of CD24. Information on bacterial mating, however, suggests that P may be present in a repressed state in this strain.

These attenuated strains appear to be immunogenic as they give good protection in the mouse protection test (*11*) and ileal loop challenge experiments in adult rabbits. This protection was also associated with the high vibriocidal antibody in the latter model.

A number of attenuated strains of cholera vibrio have previously been reported for the possible development of live oral vaccine (*13*). Some of these either failed to provide effective immunity (*3*) or reverted to toxicity (strain M13, R. A. Finkelstein, personal communication, 1976). The attenuated strains described here appear to be well suited for use in the production of live oral vaccines since they are quite stable under various conditions, able to multiply and adhere to the gut of the rabbit, provide good protection, and carry genetic markers for their identification in the environment. Such attenuated strains have the advantage of not having been exposed to mutagenic treatment. In principle, therefore, these strains should possess all the features of the wild type strain except toxin production. This is confirmed by our data.

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## RÉSUMÉ

### ATTÉNUATION DE LA VIRULENCE DE *VIBRIO CHOLERAE* PAR DES PLASMIDES P ET V: SOUCHES CONVENANT POUR L'IMMUNISATION ORALE

Dans un précédent article, les auteurs avaient rapporté que les plasmides P et V supprimaient de façon significative la pathogénicité de *Vibrio cholerae*. Dans le présent article, ils exposent une nouvelle méthode permettant d'isoler des souches atténuées de *V. cholerae* après introduction de plasmides P et V. Ces plasmides ne modifient pas l'immunogénicité des vibrions, et sont stables aussi bien *in vitro* qu'*in vivo*.

Des plasmides P et V ont été introduits dans une souche virulente KB9 par conjugaison. Plusieurs cellules porteuses de P et V ont été isolées. La virulence de ces souches a été étudiée dans des modèles animaux du choléra, et il a été démontré qu'un grand nombre d'isolats PV étaient dépourvus de pathogénicité. Par ailleurs, tandis que la souche 569B était toxinogène *in vitro*, son isolat PV (CD23) ne produisait aucune quantité décelable de toxines.

Deux souches porteuses de plasmides, KB9:PV et CD24, ont été caractérisées en détail. Dans l'épreuve de protection de la souris, la $DL_{50}$ était respectivement de 9, $2{,}7\times10^{6}$ et $4{,}2\times10^{7}$ pour les souches KB9, KB9:PV et CD24. Chez 16 lapereaux nouveau-nés infectés par voie intestinale avec les souches KB9:PV et CD24, on n'a observé ni diarrhée, ni accumulation de liquide dans le caecum. L'administration des souches KB9:PV et CD24 à un lapin adulte dans une anse iléale ne provoquait aucune perte excessive de liquide. En revanche, l'administration de la souche KB9 à des lapins adultes et à des lapereaux nouveau-nés entraînait une forte diarrhée et une perte de liquide notable.

La stabilité des plasmides a été étudiée *in vitro* et *in vivo*. L'orangé d'acridine et le dodécylsulfate de sodium n'ont pu les éliminer des cellules. Plusieurs passages sur bouillon et une incubation prolongée à 37°C étaient également sans effet. Après deux passages successifs dans une anse iléale de lapin adulte, on a recherché les plasmides dans environ 100 colonies de vibrions: aucun plasmide P et V n'avait disparu.

La protection conférée par les souches KB9:PV et CD24 a été déterminée par l'épreuve de protection de la souris et dans le modèle constitué par l'anse iléale du lapin. Dans la première épreuve, on a observé une bonne protection, notamment contre la réinfection par une souche homologue. Dans le modèle de l'anse iléale du lapin, des lapins ont été immunisés par des souches KB9:PV et CD24 vivantes, et éprouvés 35 jours plus tard. Le degré de protection se situait entre 73 et 100%, avec un titre élevé d'anticorps vibriocides. Ces deux souches sont donc immunogènes.

Les souches porteuses de plasmides étant stables et immunogènes, elles peuvent être utilisées pour la préparation d'un vaccin oral vivant, et pourraient convenir pour l'immunisation orale.


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