Persistence of an *Escherichia coli*-*Shigella flexneri* Hybrid in the Intestinal Tract of *Macaca mulatta*

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A live oral vaccine prepared from an *Escherichia coli-Shigella flexneri* 4b hybrid was administered by gavage to *Macaca mulatta*. In the three main studies involving 160 monkeys, three doses were used, generally consisting of 5 × 10^8 to 60 × 10^8 cells per dose, with an interval between doses of 6, 7, or 14 days. Rectal swab cultures at the time of the last vaccine dose, and 14 to 58 days later, revealed that the hybrid persisted in the intestinal tract for at least 7 days in 16%, and for at least 21 days in 8% of the monkeys. Our findings are comparable to those of Formal et al. for shedding of an *E. coli-S. flexneri* 2a hybrid. Protection studies with the *E. coli-S. flexneri* 4b hybrid are indicated.

Recently, investigators at the School of Aerospace Medicine isolated *Shigella flexneri* 4b from 24 of 28 cases of bacteriologically confirmed shigellosis in *Macaca mulatta*. Similarly, a recent study of 6,646 newly arrived monkeys at the National Center for Primate Biology revealed 75% of the *Shigella* isolates to be *S. flexneri* 4 (5).

From a live oral vaccine prepared from a hybrid obtained by mating a virulent *S. flexneri* 2a strain with *Escherichia coli*, Formal et al. (4) obtained significant protection against challenge with *S. flexneri* 2a. The protection was shown to be serologically specific; the vaccine was not protective against challenge with *S. flexneri* 1b or 6 (4). However, a polyvalent vaccine composed of hybrids of *S. flexneri* 1b, 2a, and 3 and *S. sonnei* I was protective against challenge with any one of the virulent parent strains (2). We know of no vaccine studies with a hybrid of *S. flexneri* 4b.

Formal et al. (3, 4) found that the *E. coli-S. flexneri* 2a hybrid, like the virulent *S. flexneri* 2a parent, could penetrate the intestinal epithelium and cause an acute inflammatory reaction; however, in contrast to the parent, the hybrid was unable to multiply and maintain a focus of infection in the lamina propria. In view of the strictly temporary parenteral residence of such a hybrid, one important factor in its capacity to protect against shigellosis may be its ability to persist in the intestine for a sufficient period to provide the necessary antigenic stimulus by repeated penetrations into the lamina propria. This paper presents observations on the persistence of an *E. coli-S. flexneri* 4b hybrid in the intestinal tract of *M. mulatta* after oral administration of living cells.

**MATERIALS AND METHODS**

**Hybrid.** The *E. coli-S. flexneri* 4b hybrid was generously supplied by Samuel B. Formal of Walter Reed Army Institute of Research, who obtained the hybrid by mating Hfr *E. coli* K-12 W1895 with *S. flexneri* 4b. The hybrid grew well on SS agar (BBL), Eosin Methylene Blue agar (EMB, BBL), MacConkey agar (BBL), and Desoxycholate agar (Difco), producing colonies indistinguishable from those of *S. flexneri* 4b strains isolated from carriers or clinical cases in these animals.

**Vaccine preparation and administration.** A 24-hr culture of the hybrid in Brain Heart Infusion broth (Difco) was massively seeded onto the surface of Brain Heart Infusion agar in petri plates. After 24 hr of incubation at 35°C, the growth was harvested into Brain Heart Infusion broth, a total of 6 to 10 ml per plate, and stored at 4°C until used later the same day. After the vaccine was administered, an unopened bottle was assayed by pipetting 0.1 ml of serial 10-fold dilutions onto the surface of Brain Heart Infusion agar and spreading with a glass elbow rod.

The vaccine was administered by gavage in 10 ml of the broth. The experimental protocol is given in Table 1. Although it was initially planned that each dose should be approximately 50 × 10^8 cells, the initial dose to the first group in the main experiments was inexplicably only 4 × 10^8. All other doses ranged from 2 × 10^9 to 60 × 10^8 cells. For the first main experiment, 44 animals were used initially, but 2 died from unrelated causes by 3 days after the last vaccine dose and were not included in the data compilation. Except for the pilot study, for which animals already on hand were used, the monkeys were newly procured *M. mulatta*.
TABLE 1. Protocol for shedding experiments

| Expt          | No. of doses | Organisms per dose | Dose interval (days) |
|---------------|--------------|--------------------|----------------------|
| Pilot         | 1            | $2 \times 10^9$    |                      |
| 1             | 3            | $0.04 \times 10^9$ |                      |
| 2             | 3            | $33 \times 10^9$   |                      |
| 3             | 3            | $10 \times 10^9$   |                      |
| Supplementary | 3            | $20 \times 10^9$   |                      |

TABLE 2. Shedding of Escherichia coli-Shigella flexneri 4b in Macaca mulatta after three doses of a live oral vaccine

| Time after last vaccine dose | Group I (42)* | Group II (39) | Group III (79) |
|------------------------------|---------------|---------------|----------------|
| day                          | 1             | 2             | 3              |
|                              | 4             | 5             | 6              |
|                              | 1             | 2             | 3              |
|                              | 4             | 5             | 6              |
|                              | 7             | 8             | 9              |
|                              | 10            | 11            | 12             |
|                              | 13            | 14            |                |
| 0*                           | --            | +             | --             |
| 14                           | --            | --            | +              |
| 21                           | +             | +             | +              |
| 35                           | +             | +             | +              |
| 42                           | +             | +             | +              |
| 49 or more                   | +             | +             | +              |

* Numbers in parentheses indicate number of monkeys in group. Shedder numbers are arbitrary.

Animals shedding.

Day of last vaccine dose.

Symbols: +, hybrid detected by rectal swab culture; --, hybrid not detected.

Three cultures, 49, 50, and 51, or 56, 57, and 58 days after last vaccine dose.

mulatta of both sexes weighing 2 to 5 kg. Animals were caged separately.

Shedding studies. Rectal swab cultures were used. In the pilot study, these were taken on each of the 3 days after administration of the single dose of vaccine. In the three main experiments, cultures were taken at the time of the last dose of vaccine and at irregular intervals thereafter (Table 2). For cultures obtained on the day of the last vaccine dose, the rectal swabs were taken just before administration of the vaccine. In the supplementary study, cultures were made on the day of the second vaccine dose and 4, 6, and 8 days after the last dose. Rectal swabs were incubated for 2 to 4 hr at 35 C in Hajna GN broth (Difco), followed by streaking to SS and EMB agars. After 24 hr at 35 C, colonies appearing to be nonfermenters of lactose were transferred to Triple Sugar Iron agar (Difco). Cultures showing a negative slant and acid butt reaction were subcultured to Motility Medium S (Difco), Urea R broth (Difco), and Sellers Citrate Mannitol agar (Difco). Motility was determined after 24 hr, and the other two media were read after 48 hr at 35 C. We have found Sellers Citrate Mannitol agar (6) of particular value for excluding E. coli paracolons from further consideration. Organisms negative in all three media were inoculated into Difco Tryptone broth for determination of indole production and maltose, xylose, rhamnose, and sorbitol broth for fermentation tests. The carbohydrates were added to Phenol Red Broth Base (Difco). Maltose was filtered through a 0.2-μm membrane filter (Millipore Corp., Bedford, Mass.); the other carbohydrate media were autoclaved. Kovac’s reagent was used to test for indole.

The hybrid reisolate was obtained from a monkey in our first experiment 51 days after the last vaccine dose; this animal had also shed the hybrid on three earlier occasions. The reisolate was used in the supplementary study only.

RESULTS AND DISCUSSION

Identification of hybrid. Biochemical differentiation of the hybrid and S. flexneri 4b was obtained by fermentation of maltose, xylose, rhamnose, and sorbitol and by production of indole. All isolates of the hybrid were positive in all five of these characteristics, and the S. flexneri 4b isolates were uniformly negative in all five. Both hybrid and S. flexneri 4b agglutinated in Shigella Poly Group B and, to a lesser extent, in Aklalescens-Dispar Group antisera (Difco). With type-specific antisera, agglutination occurred in S. flexneri 4 and in some lots of S. flexneri 3 antisera (Difco), as well as in an antisera for group factor 6 obtained from Walter Reed Army Institute of Research.

Shedding. In the pilot study, a total of 7 of the 20 animals shed the hybrid during the 3-day
period after administration of the dose of 2 × 10⁸ cells, 6 on the first day, 3 on the second, and 1 on the third. One of the seven animals shed the organism on 2 of the 3 days, and one animal shed the organism on all 3 days.

Shedding of the hybrid in the three main experiments is detailed in Table 2. Combining the groups, a total of 26 of 160, or 16%, shed. Using four doses of a S. flexneri 2a hybrid vaccine administered at 3- to 4-day intervals and culturing daily for 10 days after the last dose, Formal et al. (1) observed shedding in 22 of 24 monkeys. However, only 4 of the 24, or 17%, shed the hybrid more than 6 days after the last dose. We feel that a comparison between our findings and shedding after the 6th day in Formal's study is reasonable, since our first cultures for shedding were at the time of the last vaccine dose and therefore not earlier than 7 days since the preceding (second) dose. Furthermore, Formal et al. used four cultures after the 6th day, and we generally employed three (five in experiment 1). A second difference in the two studies was the interval between successive cultures. In the investigation of Formal et al., the interval was 1 day, whereas our interval was at least 14 days (with the exception of the last three cultures in experiment 1, all of which were done 49 or more days after the last dose).

Finally, the last culture in the study by Formal's group was 10 days after the last vaccine dose, and, in ours, it was at least 42 days after the last dose. Although in our experiments shedding was comparable to that observed by Formal et al. (1), we feel that these three differences between the two studies increase the significance of our findings.

In a second similar experiment reported in the same paper, Formal et al. (1) continued daily cultures for shedding for 30 days after the last vaccine dose. Only 2 of 12 animals shed after the 9th day and none shed after the 22nd day. In our experiments, 8% of the monkeys shed the hybrid for at least 21 days and 4% for at least 42 days. Thus, long-range shedding in our experiments also appears comparable to that observed by Formal's group.

Reasoning from our pilot study, had we cultured for shedding earlier than 7 days after the last preceding vaccine dose, a higher rate of shedding would have been expected. This conclusion was supported by the findings in our supplementary experiment with the hybrid reisolates obtained from a monkey in experiment 1 which had shed the hybrid on four occasions. With four cultures, taken at least 4 days after the last preceding dose, 11 of 20, or 55%, of the monkeys shed the variant. Whether this variant is actually more persistent than the parent hybrid in the intestinal tract of M. mulata is unknown. Since the four cultures in the supplementary experiment were done 4 days or more after the last preceding vaccine dose, we have examined the findings of Formal et al. (1) for shedding at least 4 days after the last dose; 18 of 36 monkeys, or 50%, shed. Moreover, the number of cultures in the study by Formal's group was from 7 to 27. Again, we feel that our findings on the shedding of the S. flexneri 4b hybrid are comparable to the results obtained by Formal et al. with an S. flexneri 2a hybrid. From these considerations, it is concluded that protection studies with the E. coli-S. flexneri 4b oral vaccine are indicated.

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