INFECTION AND IMMUNITY, Oct. 1981, p. 222-233
0019-9567/81/100222-12$02.00/0

Vol. 34, No. 1

Role of Chemotaxis in the Association of Motile Bacteria with Intestinal Mucosa: Fitness and Virulence of Nonchemotactic *Vibrio cholerae* Mutants in Infant Mice

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Received 4 February 1981/Accepted 19 June 1981

Contrary to earlier findings with all other in vivo and in vitro models of cholera studied, nonchemotactic vibrio mutants showed a relatively greater fitness in 5-day-old infant mice as compared with chemotactic parent or chemotactic revertant strains. This trend was manifest in the relatively greater number of nonchemotactic mutants recovered from the upper small intestine at 4 and 18 h after intragastric infection. The same trend was also revealed in the significantly greater virulence (in terms of time to death) of nonchemotactic mutants as compared with the chemotactic parent or revertant strains. Histological studies in infant mice of the penetration of chemotactic and nonchemotactic vibrios into the mucus gel of the small intestine yielded the same findings as in all other models studied, i.e., significantly greater penetration by chemotactic vibrios. There was no correlation between the relative fitness of nonchemotactic vibrios in the small intestine of infant mice and the rate of recovery of viable nonchemotactic vibrios from that site. In contrast, excellent correlation was found between the relative fitness of nonchemotactic vibrios and a decrease in the recovery of viable cells of the chemotactic strain from the small intestine. This indicates that the relatively greater fitness of the nonchemotactic vibrios in infant mice was only apparent and that the observed phenomenon was actually due to an antibacterial mechanism which prevented the accumulation of the chemotactic strains in the small intestine rather than to any stimulating effect on the nonchemotactic mutant itself. To study the in vivo fate of the inoculum in infant mice, vibrios were labeled with either ^{32}P, ^{35}S, or [^{3}H]thymidine. Specific activity determinations of the ^{32}P label were compatible with the assumption of an accelerated rate of death of the chemotactic parent strain in the small intestine. Results with the other isotopes, however, were significantly different. Indeed, the amount of radioactivity retained in the small intestine after feeding labeled bacteria correlated more closely with the isotope used than with the strain of vibrio under study. Consequently, considerable doubt must be cast on the general validity of this not uncommon technique for determining the in vivo location and the death or survival of radioactively labeled bacteria.

In view of the currently popular (and largely correct) conviction that association with the mucosa enhances the ability of bacteria to colonize body surfaces and in view of our recent findings that chemotaxis (which can promote mucosal association) increased the in vivo fitness of motile vibrios (10), it was very surprising to discover that several nonchemotactic vibrios studied showed considerably higher virulence and in vivo fitness in infant mice than their respective chemotactic parent or revertant strains. One may suspect that this apparent exception to a widely applicable rule may reveal some important principles relating to the ecological consequences of mucosal association. We therefore decided to embark on a more detailed study of the mechanisms underlying these observations. The present paper reports a detailed description of the phenomenon itself as well as some early data relevant to the identification of the nature of the mechanisms involved. The data to be presented will make it possible to rule out one of our earlier speculative hypotheses. As will be demonstrated, the phenomenon appears to be due to a relative decrease in the fitness of the chemotactic parent and chemotactic revertant strains in infant mice rather than to a relatively higher degree of fitness of the nonchemotactic

mutants, a finding which points in the direction where further investigation should be fruitful.

(The data in Fig. 1 and 2 have been presented at the 13th Symposium on Cholera of the U.S.-Japan Cooperative Medical Science Program. [Freter et al., Proceedings of the 13th Joint Conference on Cholera, The U.S.-Japan Cooperative Medical Science Program, p. 152-181, 1978. U.S. Government Printing Office, Washington, D.C.].)

MATERIALS AND METHODS

Bacterial strains. The parent and nonchemotactic mutants used in these studies are described in accompanying papers (9, 10). In addition, we used strain CA401-483 which is a nonchemotactic, smooth-swimming mutant of strain CA401, a classical Inaba strain; both CA401-483 and CA401 were kindly supplied by Neal Guentzel (University of Texas, San Antonio). Neal Guentzel states (personal communication, 1978) that the parent strain has low levels of cell-bound hemagglutinin and does not adhere to isolated intestinal brush border membranes. Strain CA401-483-R is a motile, chemotactic revertant of strain CA401-483, isolated during the present study by subculturing areas of spreading growth which appeared spontaneously in semisolid agar.

Infection of infant mice. Cultures in Trypticase soy broth without glucose (TSB; BBL Microbiology Systems) were grown for 16 h at 37°C in a rotary shaker. The bacteria were then centrifuged and taken up again in fresh TSB to 4×10^5 vibrios each of parent and nonchemotactic mutant strains per ml. In some experiments the inoculum also contained 0.01% Evans blue to facilitate tracing the inoculum in the animals, as advocated by Baselski et al. (1). The inoculum was given in 0.05-ml amounts directly into the stomach of 5- to 6-day-old BALB-Cwm mice of a strain maintained in this department by William Murphy. After infection the animals were returned to the dams until the end of the experiment.

The animals were sacrificed at intervals after infection. The entire small intestine was removed and homogenized in 25 ml of TSB in a Virtis homogenizer. In some experiments, the upper and lower halves of the small intestine were homogenized and cultured separately. Suitable dilutions of the homogenates were cultured in pour plates of semisolid agar, and viable counts of chemotactic and nonchemotactic vibrios were determined by counting colonies of characteristic morphology, as described elsewhere (9).

Infection of infant mice with radioactively labeled vibrios. Infection in infant mice with radioactively labeled vibrios was carried out as described above, with the following modifications. The bacteria were first grown overnight at 37°C on a rotary shaker in a medium consisting of 1% Trypticase (BBL) and 0.5% NaCl. A 2-ml volume of the culture was then centrifuged, and the bacteria were resuspended in 6 ml of one of the following media.

(i) For labeling with ^{35}S, the medium contained (per liter): 11.2 g of K_2HPO_4, 4.8 g of KH_2PO_4, 0.81 g of NH_4Cl, 1 g of glucose, 0.5 mg of $FeCl_3 \cdot 6\ H_2O$. To 6 ml of this medium, 0.5 mCi of $Na_2{}^{35}SO_4$ was added.

(ii) For labeling with [^3H]thymidine, the same medium was used, except that the [^{35}S]sulfate was replaced with 1 mCi of uniformly ^3H-labeled thymidine (New England Nuclear Corp.) plus 600 μg of deoxyadenosine.

(iii) Labeling with ^{32}P was done in a medium containing (per liter): 2.09 g of morpholinopropane sulfonic acid buffer, 2.0 g of $(NH_4)_2SO_4$, 0.25 g of $MgSO_4 \cdot 7H_2O$, 1 g of glucose, and 0.5 mg of $FeCl_3 \cdot 6H_2O$ adjusted to pH 7.1. To 6 ml of this medium 0.5 mCi of $NaH_2{}^{32}PO_4$ plus 10 mg of unlabeled K_2HPO_4 were added. In some experiments the amount of unlabeled K_2HPO_4 was lowered to 0.6 mg (in 6 ml of medium). This resulted in significantly higher specific activity but a lower yield of the bacteria.

All media were sterilized by filtration before addition to the centrifuged bacteria. The bacterial suspensions were kept at 4°C overnight and then incubated for 6 h at 37°C in an incubator located in a cold room. The incubator was turned on at 2:00 a.m. by an electric timer. After incubation the radioactive cultures were deposited, drop by drop, in the center of a membrane filter (Nalge no. 450-0020, 100-ml capacity, 0.22-μm pore size) which was subjected to constant suction. The deposit was then washed by successively draining 10 10-ml portions of sterile TSB through the filter. To remove isotopes which might have been merely adsorbed rather than incorporated and to readapt the labeled vibrios to the physiological state that was obtained in earlier experiments, the washed bacteria were suspended in 20 ml of sterile TSB, incubated for 1 h at 37°C, and then washed again twice by centrifugation and resuspension in sterile TSB. Considerable amounts of radioactivity (up to 30% of the total) were released by the bacteria during this final incubation. In spite of the extensive washing procedures employed, the final inocula contained between 2 and 15% of unbound radioactivity which must have been released from the washed bacteria. This proportion of free radioactivity could not be reduced by additional washings.

Specific radioactivities (counts per minute of radioactivity divided by the number of viable bacteria) of the inocula ranged from 1/3.1 to 1/58 for ^{32}P incorporated at the low concentration of unlabeled phosphate and from 1/388 to 1/914 for ^{32}P incorporated at the higher concentration of carrier phosphate. The corresponding figures for ^{35}S and ^3H were 1/60 to 1/501 and 1/39 to 1/169, respectively.

Inocula were 0.05 ml per mouse, delivered directly into the stomach, containing from 1×10^6 to 5×10^7 bacteria in different experiments and consisting of approximately equal proportions of a chemotactic and a nonchemotactic strain. The two bacterial strains in a given inoculum were labeled with different isotopes, either ^{32}P and ^{35}S or ^{32}P and ^3H. To rule out any possible effects of the different labeling procedures on the outcome, the chemotactic strain was labeled with ^{32}P and the nonchemotactic mutant was labeled with one of the alternate isotopes in approximately half of the experiments, and the opposite pattern of labeling was employed for the rest.

The mice were sacrificed 4 h after inoculation. The upper half of the small intestine was homogenized in 0.8 ml of TSB in a Teflon pestle tissue grinder. A portion of the homogenate was cultured quantitatively in semisolid agar as described above. Another portion of the homogenate (0.25 or 0.5 ml) was incubated with 5 ml of Protosol (New England Nuclear) at 56°C until completely digested (approximately 1 h), mixed with 5 ml of Omnifluor (New England Nuclear) and counted in a liquid scintillation counter. Corrections for quenching were made on the basis of counts of samples "spiked" with known activities of the isotope to be measured. The inocula (or suitable dilutions thereof) were digested and counted in exactly the same manner as specimens from the animals.

Histological studies of bacterial invasion of mucus gel. Histological studies of bacterial invasion of mucus gel were analogous to those described in an accompanying paper (10). Infant mice were given 5×10^9 each of bacteria plus an equal number of polystyrene particles (1.1-μm diameter) directly into the stomach. At various intervals thereafter the animals were sacrificed, and the upper half of the small intestine removed, frozen, sectioned, and stained as described elsewhere (10).

Statistical methods. Analysis of covariance was as described by Kmenta (13). Confidence intervals of means were calculated as described by Bowker and Lieberman (6).

RESULTS

Table 1 summarizes the results of survival and growth of nonchemotactic mutant 31 and its chemotactic parent in the small intestine of 5- to 6-day-old mice. These were derived from two different types of experiments. The data at 18 to 22 h after infection were obtained as described above with unlabeled vibrios. The corresponding data at 4 h after infection were calculated from experiments which had been designed for a different purpose (cf. below) and which involved vibrios labeled with ^{32}P, ^{35}S, or [^{3}H]thymidine (cf. above). The 4-h experiments involved a larger initial inoculum which was necessary to achieve a measurable recovery of the radioactive labels. Preliminary data (not shown) with mice given approximately 4×10^4 unlabeled vibrios and the data in Tables 2 and 3 indicate, however, that the shifts in the relative recovery rates at 4 h after infection were not affected by the radioactive labels or by the size of the inoculum.

As may be seen (Table 1), the relative fitness of the nonchemotactic mutant strain in infant mice was significantly higher than that of the chemotactic parent, the exact opposite of what had been observed earlier with these strains in other in vivo and in vitro situations (8, 10). This relative advantage of the mutant was well expressed at 4 h after infection and remained at that level until the end of the experiment. It is also noteworthy that the number of vibrios present in the upper small intestine at 4 h represented only a small fraction of the number given as the inoculum, i.e., 0.0269 of the parent vibrio and 0.167 of the mutant strain. Cultures of stomach and large intestine (not shown) indicated that most of the inoculum had passed from the stomach into the large intestine at 4 h after infection. The vibrios found in the upper small intestine at that time represent, of course, the number remaining of the original inoculum plus subsequent changes in the number resulting from multiplication or death of the bacteria. It follows that the large number of vibrios found in the upper small intestine at 18 to 22 h after infection (i.e., 26.2 or 132.2 times the number present in the original inoculum; Table 1) must represent the progeny of only a small fraction of the original inoculum, an observation which had also been made by Baselski and Parker (3). Vibrio populations present in the entire small intestine at 18 to 22 h after infection were considerably higher than those in the upper small intestine (Table 1), indicating that the density of the vibrio populations increased along the length of the small intestine.

The increased relative fitness of nonchemotactic mutants in infant mice was also reflected in their relative virulence for this animal. These experiments were conducted as described above, except that the animals were not sacrificed but were allowed to die as a result of the infection. It should be noted that vibrios were apparently confined to the gut of the infant mice at all times and could not be cultured from heart blood or liver even in moribund animals.

Figure 1 shows the cumulative death rates of mice infected with nonchemotactic mutant 31 or its chemotactic parent strain. Figure 2 presents similar data for the nonchemotactic mutant 40 and a chemotactic revertant isolated from it. As may be seen, death rates were significantly faster with the nonchemotactic strain in both instances. The infant mice used in these experiments were exquisitely sensitive to experimental cholera, i.e., the 50% lethal dose (LD_{50}) of the parent strain was less than 10 vibrios per mouse, and the inoculum of 3×10^4 vibrios used in the above experiments represents, consequently, 3,000 LD_{50}. It is therefore not surprising that, with such high infective doses, the experiments in Fig. 1 and 2 showed differences only in terms of prolonged time to death rather than permanent survival.

In view of the fact that the above experiments had been carried out with mutants derived from only one parent strain, it was of interest to determine whether the increased relative fitness of nonchemotactic mutants in infant mice could

TABLE 1. *Recovery by quantitative culture of nonchemotactic mutant 31 and its chemotactic parent from the small intestine of infant mice*

Organ cultured	Time after infection (h)	% of mutants in inoculum[a]	Inoculum size	Fraction of inoculum recovered		% of mutants in small intestine[a]	No. of mice cultured
				Parent	Mutant 31		
Upper small intestine	4	~50	1×10^6 to 5×10^7	0.0299	0.167	86.1	95
Upper small intestine	18 to 22	~50	4.3×10^4	26.2	132.2	83.6	13
Entire small intestine	18 to 22	~50	$\sim 4 \times 10^4$	108	827	88.4	97

[a] Percentage of total vibrios that are mutants.

TABLE 2. *Association of four chemotactic and nonchemotactic vibrio strains with the upper small intestine*

Mouse no.	% of inoculum recovered		Mutant 31/parent[a]	% of inoculum recovered		Mutant CA401-483/parent[a]
	Mutant 31	Parent		Mutant CA401-483	Parent CA401	
1	70.16	29.55	2.37	2.10	4.03	0.52
2	94.44	12.57	7.51	4.49	4.84	0.93
3	100.00	14.46	6.91	3.90	4.24	0.92
4	53.96	13.01	4.14	1.64	3.63	0.45
5	89.68	38.99	2.30	3.55	8.93	0.40
6	58.73	1.06	55.4	1.60	0.62	2.58
7	81.74	2.01	40.6	2.31	0.74	3.12
8	88.88	7.92	11.2	2.27	2.24	1.01
9	55.55	9.05	6.13	1.66	2.78	0.60
10	60.31	15.72	3.84	1.45	2.71	0.54
11	87.30	5.97	14.62	2.30	2.07	1.11

[a] Represents the increase in the proportion of the mutant.

TABLE 3. *Association of four chemotactic and nonchemotactic vibrio strains with the upper small intestine*

Mouse no.	% of inoculum recovered		Mutant 31/parent[a]	% of inoculum recovered		Mutant CA401-483/revertant[a]
	Mutant 31	Parent		Mutant CA401-483	Revertant	
1	34.70	19.73	1.75	4.30	2.61	1.65
2	12.47	0.92	13.55	2.84	0.30	9.47
3	14.88	1.31	11.36	1.97	0.42	4.69
4	12.58	1.90	6.62	2.82	0.20	14.1
5	10.94	0.92	11.89	1.34	0.06	19.5
6	24.11	6.71	3.59	3.13	0.48	6.52
7	12.00	1.57	7.64	2.27	0.26	8.73
8	27.64	1.64	16.85	2.27	0.18	12.61
9	25.29	2.50	10.1	3.87	0.39	9.92
10	18.88	2.23	8.46	2.94	0.59	4.98
11	13.52	1.90	7.12	2.67	0.17	15.7
12	35.29	15.78	2.23	6.32	3.45	1.83

[a] Represents the increase in the proportion of the mutant.

also be observed with mutants derived from another vibrio strain. For this reason, nonchemotactic mutant CA401-483, its chemotactic parent CA401, and its chemotactic revertant CA401-483R were studied in subsequent experiments. To insure that the individual mice used in these studies were comparable to those used earlier, simultaneous controls were provided by inoculating mutant 31 and its parent strain along with the strains under study. The procedure was as described above, with the following changes: (i) the mice were sacrificed 4 h after infection; and (ii) the inoculum consisted of four components, namely, 8×10^7 cells each of the chemotactic and nonchemotactic strains under study plus 1.5×10^5 cells each of nonchemotactic mutant 31 and its chemotactic parent. Since the latter two strains, but not the former, were resistant to streptomycin, homogenates of upper small intestines of infected animals were cultured in duplicate in pour plates of semisolid agar containing 1 mg of streptomycin per ml and

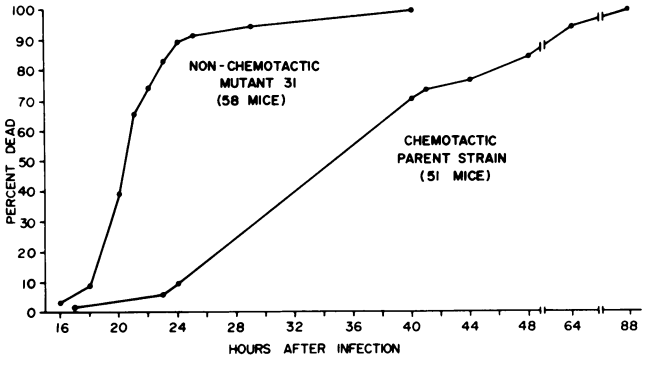

FIG. 1. *Cumulative death rates of 5-day-old infant mice inoculated intragastrically with 3 × 10⁴ vibrios.*

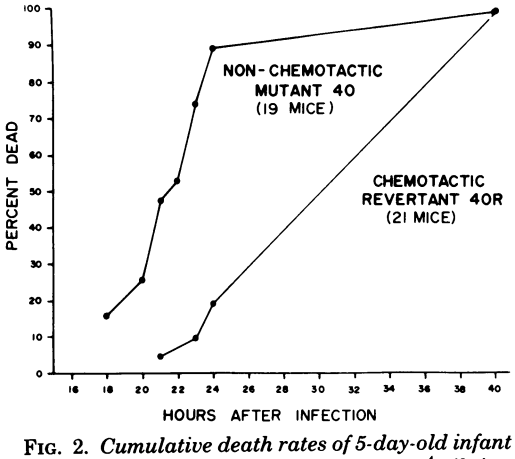

FIG. 2. *Cumulative death rates of 5-day-old infant mice inoculated intragastrically with 3 × 10⁴ vibrios.*

in the same medium without antibiotic. Only the control strains (mutant 31 and its parent) would form colonies in agar containing streptomycin. The medium without antibiotic would, of course, support the growth of all four vibrio strains. Since the control strains were approximately 500 times less numerous in the inoculum than the vibrios under test, they did not contribute greatly to the number of colonies appearing in antibiotic-free semisolid agar. Thus, by subtracting the number of colonies counted in agar containing streptomycin from those in antibiotic-free medium, precise quantitation of the vibrios under test could be accomplished.

Table 2 lists the results of one such experiment. As may be seen, the control strains (mutant 31 and its parent) reproduced the phenomenon described earlier, i.e., in every single animal the recovery of the nonchemotactic mutant (expressed as a percentage of the original inoculum) was considerably higher than that of the parent. In contrast, the recovery of nonchemotactic mutant CA401-483 in these same mice was comparable to that of its parent. These results were quite different, however, when nonchemotactic mutant CA401-483 was compared with the chemotactic revertant CA401-483-R derived from it (Table 3). In these experiments, the recoveries of nonchemotactic mutant 31 and nonchemotactic mutant CA401-483 were superior to their respective chemotactic parent or revertant counterparts by similar margins. One must conclude, therefore, that the observation in infant mice of superior fitness of nonchemotactic mutant strains derived from a single parent can be reproduced as well with a strain of different ancestry. The failure to observe this phenomenon when mutant CA401-483 was compared with its parent CA401 can be explained by postulating that this mutant differed from its parent in more than one characteristic. This explanation has recently been confirmed by Guentzel (personal communication, 1981) who noted that mutant CA401-483 (which had been selected after mutagenesis with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine) has somewhat reduced motility and virulence as compared with its parent CA401, a defect which he had not yet discovered when he sent the strain to our laboratory in 1978.

The superior relative fitness of the chemotactic parent observed in earlier studies has been correlated with its greater ability to associate with intestinal mucosa (10). It was of obvious interest, therefore, to determine whether its relatively inferior fitness in infant mice was paralleled by a decreased ability to interact with the gut mucosa of this animal. For this purpose infant mice were inoculated directly into the stomach with a mixture of 5×10^9 polystyrene spheres plus an equal number of either nonchemotactic mutant 31 or its parent strain. The animals were sacrificed at intervals thereafter, and frozen sections of upper small intestine were prepared as described above.

Sections of tissue obtained 30 min after feeding from mice given nonchemotactic mutant 31 showed numerous vibrios and polystyrene particles in the lumen. Some intervillous spaces also contained large numbers of particles and bacteria, but there were no intervillous spaces filled only with bacteria in the absence of particles. We interpret this to mean that mutant 31 was no more efficient in penetrating intervillous spaces than the inert particles. In contrast, sections from mice fed the chemotactic parent strain 30 min before sacrifice showed numerous intervillous areas filled with large numbers of vibrios, but without particles. This indicates that the parent strain was considerably more efficient in penetrating intervillous spaces than were the inert particles. Apparently, then, the superior ability of the chemotactic parent to associate with mucosa noted in earlier studies (8, 10) was also demonstrable in infant mice. We must admit that this finding invalidates an earlier speculative hypothesis of ours, which postulated that chemotactic vibrios might be drawn into the intestinal lumen of baby mice by a taxin gradient emanating from the coagulated milk within the lumen.

Sections prepared from mice sacrificed 60 min after inoculation with vibrios and particles were similar to those prepared at 30 min. When mice were sacrificed 2 h after feeding, very few particles or vibrios could be seen in the lumen or the intervillous spaces. This confirms the data presented above which indicate that only a small fraction of the original bacterial inoculum remains in the small intestine. The finding is also consistent with in vitro results obtained earlier (8) showing that the association of vibrios with the mucus gel of intestinal slices is reversible to a considerable extent.

The increased relative fitness in infant mice of the nonchemotactic mutant vibrios studied may have been due to either a stimulatory effect on the mutants or an inhibitory mechanism acting on the chemotactic parent or revertant. The types of experiments described above do not permit a distinction to be made between these alternatives. The following experiments were designed to remedy that shortcoming. The rationale was to make use of the fact that the relative fitness of nonchemotactic mutants differed considerably in magnitude in individual infant mice. For example, in mouse no. 6 of Table 2 the number of mutant 31 recovered from the upper small intestine (58.73% of the number inoculated) was more than 55 times greater than the recovery of its parent (1.06% of the number inoculated). In contrast, in mouse no. 1 (Table 3) the recovery of mutant 31 was only 1.75 times higher than that of its parent. It was reasoned, therefore, that whatever mechanism is responsible for the relatively greater fitness of mutant 31 must be more active in mice in which this microorganism shows a higher degree of relative fitness, and vice versa. The in vivo increase in the proportion of mutant to parent was chosen as a measure of relative fitness. It was defined as A/B, with A = (number of mutants recovered from intestine)/(number of mutants in the inoculum) and B = (number of chemotactic vibrios recovered from intestine)/(number of chemotactic vibrios in inoculum).

The results shown in the following figures are analyzed by plotting the logarithm of A/B, i.e., of the in vivo change in the proportion of the nonchemotactic mutant for each individual infant mouse as the independent variable (i.e., along the horizontal axis). This is compared with various other parameters that one might suspect of being involved in causing this change which are plotted as the dependent variables along the vertical axis. In 12 experiments of this type a total of 105 mice were studied. Only 95 of these could be evaluated for the parent strain, because the radioactive label was too low in the rest. Briefly, the infant mice were given a mixture containing equal portions of mutant 31 and its parent strain, each labeled with a different radioactive isotope. At 4 h after infection viable counts and radioactivity in the upper half of the small intestine were determined as described above.

Figure 3 presents data in which the dependent variable (i.e., the variable plotted along the vertical axis) is the logarithm of the in vivo change of the specific radioactivity of the parent strain (note that the independent variable in this and in all subsequent figures is always the same, i.e., the in vivo change in proportion of viable counts of mutant 31, regardless of whether the dependent variable is derived from the parent or the mutant). The in vivo change in specific radioactivity is defined as the specific radioactivity of the bacteria in the inoculum divided by their specific activity when subsequently recovered from the gut: Change in specific radioactivity = S (inoculum)/S (mouse), with S = (radioactive counts per minute)/(viable counts).

Others have interpreted changes in specific radioactivity as indicative of growth or death of the bacteria: as the bacteria multiply the viable count increases while the radioactivity remains the same, such that the change in specific radioactivity as defined above becomes larger than 1 (and its logarithm [Fig. 3] becomes larger than zero). Conversely, a value less than 1 (negative logarithm in Fig. 3) for this parameter has been

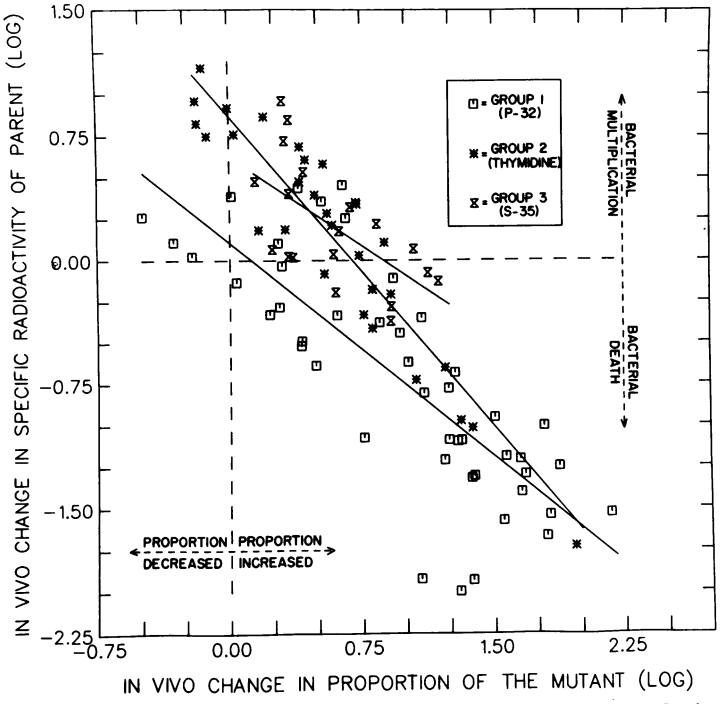

FIG. 3. *Correlation between relative fitness of the nonchemotactic mutant (i.e., the in vivo change in its proportion) and the in vivo change in specific radioactivity of the chemotactic parent strain in the upper small intestine. Symbols differentiate between the isotopes used in labeling the parent strain. Each symbol represents one mouse.*

taken to indicate bacterial death. The tacit implication underlying this interpretation is, of course, that all radioactivity remains associated with the bacteria in vivo.

If the data for group 1 (phosphate-labeled parent strain) in Fig. 3 had been the only ones available, they would have tempted us to come to a rather firm conclusion: the linear regression line for this group intersects very close to the origin, suggesting that in those animals in which the proportion of the mutant increased, there was a proportional rate of death of the parent strain and vice versa. Unfortunately, the data obtained when the parent strain was labeled with one of the other isotopes (groups 2 and 3, Fig. 3) do not confirm such a conclusion. Analysis of covariance revealed that the linear regression lines differed significantly in slope (P = 0.0106 for group 2 versus group 1 and not significant for group 3 versus group 1) as well as in the intercept of the x axis, i.e., of the dotted horizontal line at no change in specific activity in Fig. 3 (P = 0.0000 for group 2 versus group 1, and P = 0.0122 for group 3 versus group 1). In other words, the apparent in vivo change in specific radioactivity of the parent strain differed significantly depending on the nature of the radioactive label used in these experiments, a feature which cautions against overly liberal interpretation of such data. Figure 4 shows similar data for the in vivo change in specific radioactivity of mutant 31. As may be seen, there was no correlation whatever of this parameter with the change in proportion of the mutant.

Figures 5 and 6 are analogous to Fig. 3 and 4, relating as the dependent variable the number of bacteria recovered from the mouse intestine, normalized as a percentage of the inoculum. As is apparent, the only parameter which correlated strikingly with the in vivo change in the proportion (fitness) of the nonchemotactic mutant was the recovery of viable cells of the parent strain (Fig. 5). No correlation existed between fitness and (i) recovery of viable cells of the nonchemotactic mutant (Fig. 6), (ii) recovery of radiolabel of the chemotactic parent (not shown), and (iii) recovery of radiolabel of the nonchemotactic mutant (not shown).

DISCUSSION

A number of investigators have used radioactively labeled bacteria in experiments designed to determine the in vivo retention or the in vivo death and multiplication rates of vibrios and

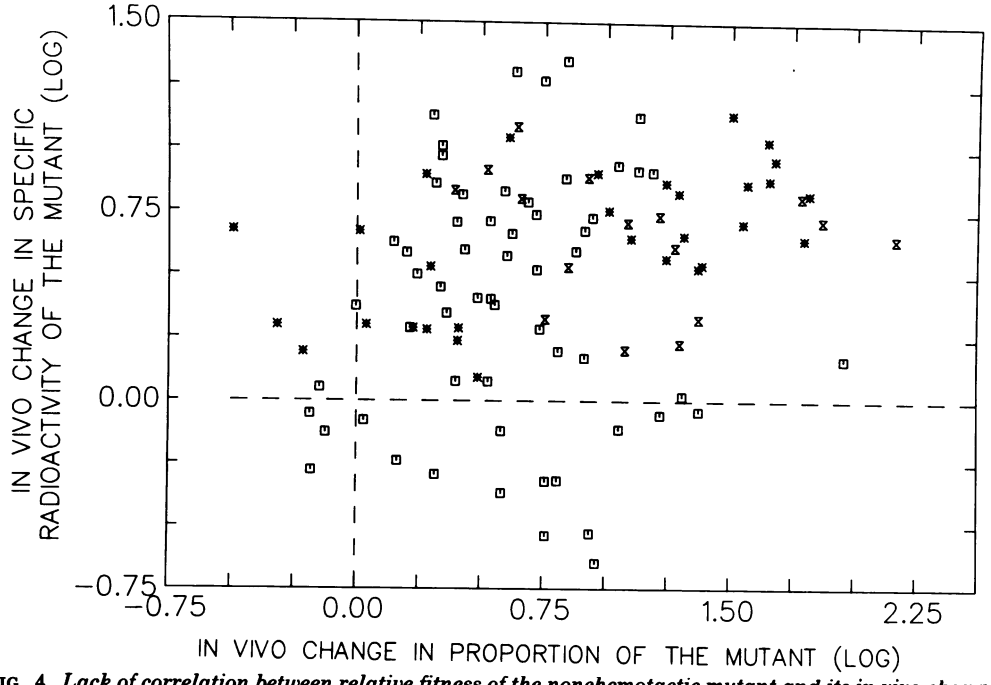

FIG. 4. *Lack of correlation between relative fitness of the nonchemotactic mutant and its in vivo change in specific radioactivity in the upper small intestine. Symbols (same as in Fig. 3) refer to isotopes labeling the nonchemotactic mutant.*

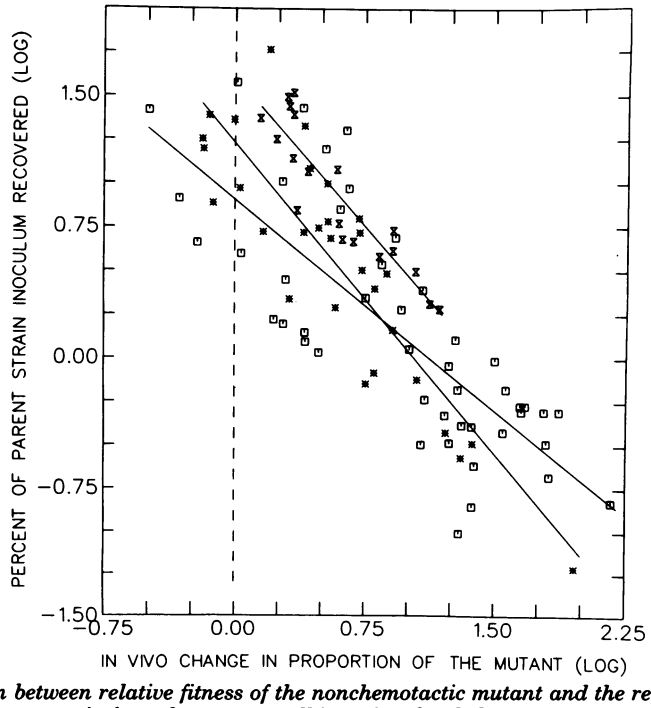

FIG. 5. *Correlation between relative fitness of the nonchemotactic mutant and the recovery of viable cells of the chemotactic parent strain from the upper small intestine. Symbols represent the same groups of mice as in Fig. 3.*

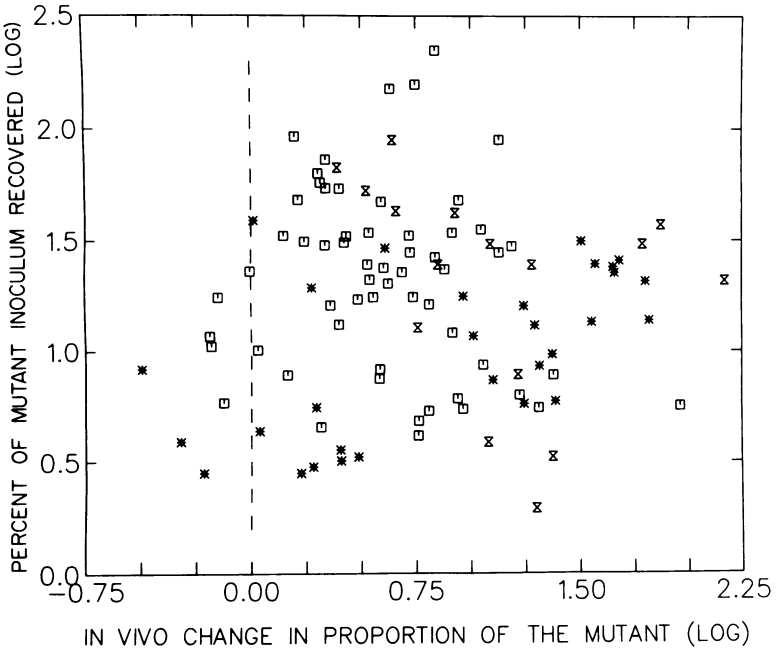

FIG. 6. *Lack of correlation between relative fitness of the nonchemotactic mutant and its recovery as viable cells from the upper small intestine. Symbols represent the same groups of mice as in Fig. 4.*

other microorganisms (e.g., 2–4, 11, 12, 14, 15, 17–19). Our data cast considerable doubt on the general validity of this not uncommon experimental approach and, for this reason, have been described in some detail in this paper. As mentioned above, the inocula of radioactive vibrios contained between 2 and 15% of free radioactivity which could not be removed by further washing. Cultivation in TSB of these bacteria increased the proportion of free radioactivity still further, pointing to an equilibrium between free and cell-bound radioactivity. This is in agreement with the data of Green and Goldstein (11) who noted that the unbound ^{32}P in their labeled bacterial inocula increased from an initial 9.8% to 21.7% of total radioactivity after 4 h. Since the degree of retention of radioactive compounds released by bacteria at in vivo sites depends on the type of molecule into which the isotopes are incorporated and since different isotopes are likely to be released in different chemical forms, one must expect that the amount of radioactivity that is released in vivo by the bacteria and subsequently retained by the animal tissue will differ with the isotope involved. This was indeed the case in the present study. The data in Table 4 indicate that the recovery rates of the three radioactive labels from the gut differed significantly from each other. In contrast, recovery rates for any one label were similar, regardless of whether the label was on the parent or the mutant strain. The only simple explanation for these findings is to assume that most of the radioactivity recovered from the mouse gut represented isotopes which had been released from the bacteria when the bulk of the inoculum passed through the lumen of the small intestine and which were in a chemical form that caused them to be bound by the gut tissue. In other words, the amount of radioactivity retained in the small intestine was not a reliable indicator of the number of inoculated bacteria retained at that site. This is supported by the data of Knop and Rowley (14), who found that most of the radioactivity released from the gut of mice exposed to ^{32}P-labeled vibrios no longer sedimented on centrifugation within 10 min. The same conclusion can be reached from the data in Table 4. If one assumes that all [^{3}H]thymidine

TABLE 4. *Recovery of various isotopes from the small intestine of mice 4 h after feeding labeled vibrios*

Label	Strain	*n*	% of radioactivity recovered	95% confidence interval
^{32}P	Parent	59	7.28	6.23–8.51
	Mutant 31	46	8.54	7.49–9.73
[^{3}H]thymidine	Parent	30	2.56	2.16–3.02
	Mutant 31	30	2.25	1.97–2.57
^{35}S	Parent	19	5.44	3.91–7.57
	Mutant 31	16	4.47	3.15–6.34

recovered from the small intestine of the mice (2.25 to 2.56% of inoculum, Table 4) is still completely associated with bacteria, then one would expect the same percentage of recovery for the other labels as well. Since recovery of ^{32}P label in these experiments was 7.28 to 8.54% (Table 4), this label must represent a minimum of 70% free radioactivity. Unfortunately, most of the workers quoted above did not report the amount of free radioactivity in their inocula and merely assumed that this would not be an important factor. These considerations do not rule out the possibility that contamination of bacterial inocula with a small amount of free radioactivity may be of minor significance in experiments where the bulk of the bacterial inoculum is to be traced (e.g., in reference 19). The situation is quite different, however, when data are to be gathered on a small fraction of the original inoculum that has been retained in a given site (as in the present study and many of the others quoted above). In such circumstances, preferential retention of unbound radioactivity may lead to a situation where the retained free radioactivity exceeds the radioactivity of the retained bacteria, thus rendering interpretation impossible. When data of this nature are evaluated in terms of bacterial retention, death, or survival, they must therefore be rejected unless additional controls are provided to prove that the radioactivity recovered from in vivo sites is indeed still associated with the microorganisms.

The finding that chemotactic vibrios had decreased virulence in infant mice (in terms of time to death) was totally unexpected in view of all earlier data we had obtained in vivo and in vitro (8–10). However, subsequent quantitative studies of vibrio populations in the upper small intestine of infected infant mice were consistent with this observation: when a total of 10^6 to 10^7 vibrios (half of them chemotactic, the other nonchemotactic) were fed to infant mice, an average of 16.7% of the inoculum was recovered 4 h later from the upper part of the small intestine (Table 1). The rest of the inoculum had either died or passed on to lower regions of the gut (the stomach contained few or no bacteria at this time). Among the bacteria recovered from the small intestine there were strikingly fewer chemotactic than nonchemotactic vibrios, i.e., the proportion of nonchemotactic to chemotactic vibrios was considerably increased relative to the inoculum. The obvious alternative explanations for this observation are as follows: (i) The nonchemotactic mutant had relatively greater fitness in infant mice. "Fitness" is an ecological catch-all term which in its present application reflects various parameters such as the rate of retention of the vibrios on the mucosa, their rate of multiplication, and their rate of death in the animals. Alternatively, (ii) it is possible that the nonchemotactic mutant grew normally in this animal, but that infant mice had a defense mechanism (that was lacking in the other animal models studied), which selectively or at least predominantly affected the growth or survival of the chemotactic vibrios. To distinguish between these alternatives, the experiments presented in Fig. 3 through 6 were based on the simple rationale that an obvious correlation should become apparent when the relative fitness of the nonchemotactic mutant, i.e., the in vivo change in its proportion, is plotted for each mouse against the parameter that is responsible for this change. It was therefore rather surprising to find that no such correlation existed between the in vivo change in the proportion of the mutant and the fraction of the original mutant inoculum that was recovered from the small intestine at the end of an experiment (Fig. 6). This finding does not support the first alternative, namely, that the relatively superior in vivo fitness of the mutant might have been due to its superior retention or rate of survival or multiplication in the small intestine of the infant mouse. In contrast to this, an excellent negative correlation existed between the fraction of the original parent inoculum that was recovered from the upper small intestine at the end of an experiment and the in vivo change in the proportion of the mutant (Fig. 5). In other words, Fig. 5 shows that mice which harbored a relatively high proportion of the mutant at the end of the experiment showed a correspondingly lower recovery of the parent strain and vice versa. This finding strongly supports the second alternative, namely, that the relatively inferior fitness of the chemotactic parent in infant mice was due to a reduced rate of retention or multiplication (or both) of the parent strain in the small intestine of this animal (and not to any superior quality of the mutant). In view of our histological findings mentioned above, which show that the parent strain was more efficient than the nonchemotactic mutant in penetrating the mucus gel of the infant mouse small intestine, it seems unlikely that its retention at this site was inferior to that of the mutant. Consequently, it is most consistent with the available data to assume that there was an antibacterial (bacteriostatic or bactericidal) mechanism in the deeper layers of the intervillous spaces of the infant mouse intestine. Since chemotactic vibrios are attracted preferentially toward these deeper areas, they would then be at a disadvantage relative to nonchemotactic vibrios. It should be noted that antibacterial mechanisms of intestinal mucosa that are active against chol-

era vibrios have previously been described in infant mice (5, 16) and other animals (7). In summary, we speculate that populations of both types of vibrios may multiply at comparable rates in the more superficial layers of the intestinal mucus gel, but that a substantial proportion of the chemotactic strain (but not the nonchemotactic mutant) constantly penetrates into the deeper mucosal layers where they are either severely retarded in their growth or actually killed by the hypothetical antibacterial mechanism which we postulate to be located at that site. Direct proof for this working hypothesis will require a demonstration of in vivo differences in growth rates between chemotactic and nonchemotactic vibrios in infant mice. Unfortunately, the determinations of specific radioactivity which in the past have been used by others for this purpose were found to be unsuitable for such application. Perhaps the use of segregating genetic elements, a technique pioneered by Meynell and Subbaiah (16), may be more reliable, and we plan to make attempts toward the development of a suitable system.

The data presented also indicate rather strongly that the differences in fitness and virulence between the chemotactic and non-chemotactic vibrios studied were indeed due to differences in chemotactic responsiveness, rather than to redundant mutations unrelated to chemotaxis. As described above, the phenomenon could be demonstrated with three nonchemotactic mutants and two of their revertants. Moreover, Guentzel (personal communication, 1981) reports that he has isolated a total of five mutants (without the use of mutagenesis) from our parent strain and from strain CA401, all of which caused a significantly shortened time to death in infant mice as compared to their respective parent strains, (i.e., they reacted essentially as shown for our mutants in Fig. 1 and 2). It is highly unlikely that all of these mutants which consistently showed increased fitness in the infant mouse carried an identical secondary mutation unrelated to chemotaxis. On the other hand, the present data do not rule out the possibility that the nonchemotactic strains differed in some characteristic which is phenotypically linked to chemotaxis. This possibility is also unlikely, however, because the present data indicate strongly that the apparent increase in fitness of the nonchemotactic mutant was actually caused by an antibacterial mechanism that affected the parent preferentially and not to any enhanced fitness of the mutant. It should be realized in this connection that other chemotactic strains of *Vibrio* or other genera may not be susceptible to the adverse conditions on the mucosa of infant mice, and such strains, therefore, may be expected to have a greater degree of fitness in these animals than nonchemotactic mutants selected from them.

In summary, then, data reported in the present paper suggest strongly that, contrary to common assumption, mechanisms such as chemotaxis which promote mucosal association may not always function to the benefit of the invading bacterium. In the infant mice used for the present study the postulated antibacterial mechanism was not ultimately protective, in that it caused a significant increase in the time to death but did not prevent the eventual fatal outcome. It is quite possible, however, that a similar mucosal antibacterial mechanism may make the decisive difference in the presence of additional partially protective host defense mechanisms such as, for example, local immunity. It is interesting to note that antibacterial mechanisms have also been noted on other mucosae (e.g., reference 17) and one may therefore expect that the significance of this phenomenon is not necessarily limited to the gut.

ACKNOWLEDGMENT

This work was supported by Public Health Service grants AI 07631 and AI 15279 from the National Institute of Allergy and Infectious Diseases.

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