Association of salmonella mutants with germfree rats: Site specific model to detect carcinogens as mutagens

(gnotobiotic rats/carcinogen activation/host-mediated assay)

LARRY A. WHEELER, JOHN H. CARTER, FRANCES B. SODERBERG, AND PETER GOLDMAN*

Department of Pharmacology, Harvard Medical School, Beth Israel Hospital, Boston, Massachusetts 02215

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ABSTRACT  An association of the histidine auxotroph of Salmonella typhimurium (strain TA1538) within the gastrointestinal tract of otherwise germfree Sprague-Dawley rats is maintained during periods of observation lasting as long as 7 months. The bacteria are found at levels exceeding 10^8 per g in the cecum and at levels greater than 10^9 per g in the lower bowel and in the feces. Only approximately 10^4 bacteria per g are found in the posterior stomach and in the upper small intestine. The association of the salmonella mutants is maintained when the bacterial association is increased by the addition of other bacterial characteristic of the gastrointestinal flora. Carcinogenic amines, which cause strain TA1538 to revert to histidine independence in Ames' in vitro assays, increase the number of revertants in the feces when fed to the salmonella-associated rats. In contrast, the number of revertants in the feces does not increase when the rats are fed structurally related compounds which are not mutagenic to the bacteria in vitro and for which no evidence of carcinogenicity exists. Sacrifice of rats after feeding the carcinogen 2-nitrofluorene indicates that the number of revertants is increased in the cecum and colon as well as in the feces. The apparent proximity of the bacterial mutagenic response to the location of the tumor response in the colon suggests that the rat associated with the histidine auxotroph may provide a useful model for further investigation of the possible association between bacterial mutagenesis and carcinogenesis within the gastrointestinal tract. In addition, with this model it may be possible to evaluate selectively the effects of various constituents of the flora on the activation of compounds provoking the revertant response.

Concern about exposure to possible mutagenic and carcinogenic agents has stimulated the development of test systems to warn of the presence of such compounds in the environment. Since alterations in nucleic acids are the basis of mutagenesis, and possibly also of carcinogenesis (1), it seemed logical to test compounds in systems previously developed for bacterial genetics in which alterations in nucleic acid structure might be discerned on the basis of easily recognizable phenotypic alterations. Thus, alterations of DNA (2), the detection of mutations in bacteria (3) and phages (4), and the expression of prophages (5) have each served as the basis for identifying chemicals with mutagenic properties. Recently there has been particular interest in the histidine auxotrophs of Salmonella typhimurium developed in Ames' laboratory which, on the basis of reversion to histidine independence, exhibit particular sensitivity to various carcinogenic agents (6, 7).

The value of an exclusively bacterial test system is limited, however, since many compounds are not mutagenic or carcinogenic until they have been transformed to reactive molecules by mammalian enzymes (8). An initial attempt to solve this problem was the development of a host-mediated assay in which compounds to be tested are administered to a mouse whose peritoniem had been seeded with histidine auxotrophs of S. typhimurium (9). The host-mediated activation of the carcinogen can now be accomplished by the addition of microsomal preparations from liver to the in vitro bacterial test system (10).

The introduction of mammalian enzymes to the in vitro assay only supersedes the animal-mediated bacterial assay with regard to the need for mammalian activation of carcinogens. It therefore overlooks the possibility that the intestinal microflora (11, 23) and mammalian organs other than liver may carry out significant transformation of carcinogens. In addition, an animal-mediated system surpasses an in vitro system by offering the possibility of using the bacterial response to indicate the presence of labile reactive compounds at the site in the animal where these bacteria are located. A sensitive detection system of this kind might also serve to identify weak carcinogens that would escape detection in current experimental models of carcinogenesis. For example, it has been suggested that the association between the prevalence of colon cancer and the character of the diet (12) may be due to the presence of weak carcinogens arising as the result of the bacterial metabolism of compounds normally present in the animal (13). The testing of this hypothesis, which relates bacterial metabolism and mammalian physiology, is a challenging task which would likely require large numbers of animals under observation for prolonged periods of time. Under such circumstances a preliminary experiment might be justified in which conditions were sought to elicit a mutagenic response in bacteria within the colon. For this purpose we now report the association within the gastrointestinal tract of germfree rats of a strain of S. typhimurium containing a frameshift mutation in the histidine operon. This mutant, which has been designed to detect mutagenic events by reversion to histidine independence, has been found to associate throughout the gastrointestinal tract and to revert in situ in response to the addition of carcinogens to the diet.

MATERIALS AND METHODS

Chemicals. 4-Nitrophenyl, 4-nitrobenzoic acid, 2-nitrofluorene, acetanilide, and 2-acetylaminofluorene were purchased from Aldrich Chemical Co., Milwaukee, Wisc.; 3,2'-dimethyl-4-aminobiphenyl from K and K Rare Chemicals, Plainview, N.Y.; and 7-hydroxy-2-acetylaminofluorene from the Bader Library of Rare Chemicals, Milwaukee, Wisc. Azoxythane was a gift from the Division of Cancer Cause and Prevention, National Cancer Institute. Other chemicals were obtained from Sigma Chemical Co., St. Louis, Mo.

Animals. Germfree male rats of the Sprague-Dawley strain (45-47 days old) and sterilized germfree rat-mouse 7RF diet were purchased from Charles River Breeding Lab-
Medical collection of rats were potentially carcinogenic. Berkeley), had been grown in nutrient broth (Difco Laboratories, Detroit, Mich.) supplemented with 0.5% NaCl at 37°C to a density of approximately 10^8 organisms per ml. The isolation, characterization, and association of other bacteria characteristic of the gastrointestinal tract that were used in these experiments have been described (14).

Feeding of Chemicals. Compounds were fed at dosages selected, wherever possible, on the basis of previous studies that were designed to determine the carcinogenicity of these compounds. Appropriate amounts of acetanilide (15, 16), 2-acetylaminofluorene (17), 2-nitrofluorene (17), 4-nitrophenyl, 7-hydroxy-2-acetylaminofluorene (18, 19), and 4-nitrobenzoic acid were dissolved in acetone, and 3,2'-dimethyl-4-aminobiphenyl (20, 21) was dissolved in a mixture of acetone/ethanol (1:1 vol/vol). These solutions were sterilized by filtration through a 0.2 μm fluoropore filter (Millipore Filter Corp., Bedford, Mass.). A solution of azoxymethane (22) was prepared in 0.85% sodium chloride and filtered through a 0.45 μm HAWP filter (Millipore). Sterilized solutions (0.5–1.0 ml) were passed into the germfree isolators and mixed with approximately 10 g of powdered diet. The volatile solvents were allowed to evaporate for 1 hr before the animals were given access to the mixture.

Estimation of Bacteria in Gastrointestinal Segments and Feces. Rats were removed from their sterile isolators and sacrificed by cervical dislocation. The peritoneal cavity of each rat was exposed by aseptic technique, and 0.5–to 1.0-g samples of eight segments of the gastrointestinal tract were removed. The segments were described as follows: forestomach (squamous epithelium), stomach (columnar epithelium), duodenum (starting immediately posterior to pyloric sphincter, and including the bile duct junction), small intestine I (initiating 10 cm from stomach), small intestine II (initiating 55 cm from stomach), small intestine III (initiating 7 cm anterior to the ileocecal junction), cecum (blind end), and colon (initiating 5 cm from the cecum). The segments were placed in preweighed sterile tubes containing glass beads and minced with scissors; 0.85% NaCl was added to a final dilution of 1:10 (weight/volume). Less than 6 min was required from the time the animals were sacrificed to the time that the final intestinal segment was suspended in diluting fluid. The tubes were shaken vigorously on a Genie Vortex Mixer (Fisher Scientific, Boston, Mass.), and appropriate dilutions were made in 0.85% NaCl. The diluted and undiluted samples were plated on nutrient agar (6) to determine the total number of bacteria (strain TA1538 and his^+ revertants) in the sample, and on minimal medium with excess biotin (6) to determine his^+ revertants.

Bacteria of strain TA1538 and his^+ revertants were enumerated in fresh fecal samples that had been passed out of the sterile isolators in screw cap vials. Fecal samples (1.0 g) were diluted 1:10 (weight/volume) in sterile 0.85% NaCl and the procedure was followed for estimating the number of bacteria in gastrointestinal segments. The enumeration in the feces and in the gastrointestinal tract of other bacteria that were associated with the salmonella-associated rat in some experiments has been described (14).

Assay for Chemical Mutagenesis. The capacity for chemicals to revert strain TA1538 in vitro, either directly or with the requirement of an "S-9" liver homogenate fraction, was determined according to the method of Ames et al. (7, 10). Chemicals were assayed at a level of 50 μg per plate (7).

RESULTS

Association of germfree rats with strain TA1538

The sensitivity of S. typhimurium strain TA1538 to mutagenesis has been demonstrated by in vitro tests both with direct acting carcinogens (7) and with carcinogens causing mutagenesis only in an assay system fortified with a liver microsomal preparation (10). Hence mutant strain TA1538 seemed satisfactory to use in the initial attempt to associate histidine auxotrophs of S. typhimurium with germfree rats. Within 48 hr after addition of strain TA1538 to the food of the germfree rats, the mutants were present in the feces at a level of 10^9 to 10^10 per g. This association has now been successfully accomplished in all of the 23 rats in which it has been attempted. In each case the association has been maintained for as long as the rats were observed, which in some instances was as long as 7 months. In 23 rats the average number of bacteria of strain TA1538 was 2.0 × 10^8 ± 1.3 × 10^8 (SD) bacteria per g of feces; his^+ revertants of strain TA1538 are also found in the feces, the level in the 23 rats having an average value of 250 ± 180 (SD) revertants per g of feces. The deep rough mutation in strain TA1538 is not lost during association since sensitivity to deoxycholate and crystal violet (7) are retained. In addition, the rats suffer no noticeable ill effects from the association with this strain of bacteria.

The revertant response

In order to determine whether germfree rats associated with strain TA1538 can be used in a host-mediated assay for the detection of carcinogens, the density of his^+ revertants in the feces was examined in response to the feeding of various carcinogens. Fig. 1 shows a typical experiment in which the response to the feeding of the carcinogen 4-nitrophenyl is compared to that with 4-nitrobenzoic acid, an aromatic compound containing a nitro group which has not been reported to be carcinogenic.

The number of his^+ revertants in the feces is expressed in two ways in Fig. 1. When normalized in terms of the total number of mutant bacteria, there is only a minimal and transient rise in the number of revertants in response to the feeding of 4-nitrobenzoic acid (25 mg/day). There is, however, a pronounced and prolonged response in the number of revertants when the carcinogen 4-nitrophenyl (25 mg/day) is added to the diet. When the number of revertants in the feces returns to the initial level, this response is demonstrated a second time when the rats are again challenged with 4-nitrophenyl (50 mg/day). One of the rats in Fig. 1 failed to exhibit an increased number of revertants per gram of feces in response to the feeding of 4-nitrophenyl. However, the revertant response was detectable in this instance when the number of revertants was normalized in terms of
the total number of mutants in the feces. Furthermore, the revertant response occurred in these rats and consistently in four other rats when 4-nitrophenyl was fed at a level of 50 mg/day.

A similar revertant response occurs in all rats fed the carcinogens 2-nitrofluorene and 2-acetylaminofluorene and in some rats fed 2,3’-dimethyl-4-aminobiphenyl and azoxymethane; these results and others are summarized in Table 1. In this tabulation a compound was said to give a positive revertant response in a rat if at least two fecal samples obtained at intervals of 2–3 days showed a rise to at least 1000 revertants/g (a value at least twice that observed prior to feeding of the compound). It is possible that results with azoxymethane would have been more reproducible if this compound were administered by the parenteral route (22) to assure its absorption.

No response in terms of the number of his+ revertants was noted in tests with 7-hydroxy-2-acetylaminofluorene, 4-nitrobenzoic acid, and acetanilide, compounds for which no evidence of carcinogenicity exists. With these compounds the revertant response never exceeded 1000 revertants/g (values less than 520/g prior to feeding) in at least four determinations over a 7-day period after the initiation of the feeding of the compound. Thus, among the limited number of compounds examined so far, a revertant response in the germfree rat associated with strain TA1538 occurs exclusively with compounds known to be carcinogenic.

Table 1 also shows data on the revertant response with strain TA1538 to the test compounds in Ames’ original pour plate assay and in the assay utilizing activation with rat liver homogenate. Compounds not having a revertant response in the in vitro assays were also found to be negative when these assays were performed with strains TA1535 and TA100. In general, the response in the salmonella-associated rat tends to correlate in a qualitative manner with the results of the in vitro liver activated system. However, the compound 7-hydroxy-2-acetylaminofluorene for which, in limited testing, carcinogenesis has not been demonstrated (18, 19) gave a response in the liver activated in vitro assay but not in the assay with the salmonella-associated rat. The possibility has not been excluded that the response noted in the pour plate assay is caused by a reactive contaminant of 7-hydroxy-2-acetylaminofluorene.

Association of mutants and revertants in the gastrointestinal tract

Rats sacrificed several months after their association with strain TA1538 show colonization by the salmonella mutants throughout the gastrointestinal tract. Relatively high numbers of the salmonella are found in the forestomach and in
the lower bowel (Table 2). No significant change in colonization of salmonella within the gastrointestinal tract is observed when rats were fed the carcinogen 2-nitrofluorene prior to their sacrifice. However, the revertant response at various levels of the gastrointestinal tract appears to be influenced by the feeding of the carcinogen. The number of revertants is elevated over control values in the colonic and cecal contents as well as in the feces of carcinogen-fed rats (Fig. 2). Furthermore, there appears to be a correlation between the number of revertants in the feces and in the contents of the cecum and colon. Colonic contents show a consistently slightly lower number of revertants than the feces on a weight basis, a phenomenon which may be due to multiplication of revertants after the feces have been passed, but also may be due to the concentration of the feces as a result of the flow of filtered air in the germfree isolators.

Revertant levels in the feces of control rats in the experiments described in Table 2 and Fig. 2 were higher than those in the newly associated rats used in most other experiments. This is due to the prior exposure of these rats to some of the carcinogens listed in Table 1 and the gradual slight rise in the baseline number of revertants after the termination of the acute revertant response. However, the elevated number of revertants due to this effect would only tend to decrease the differences observed between control and carcinogen-fed rats and therefore does not invalidate the conclusion that fecal levels correlate with those in the colon and cecum. No correlation was observed between the revertant level in the lower small intestine and that in the feces. There was also no discernible difference between control and carcinogen-fed rats with regard to the number of revertants in the forestomach, the average for both groups being about 700 revertants/g.

In the posterior stomach and the upper small intestine where there are small numbers of S. typhimurium mutants, technical limitations make it impossible to enumerate revertants. This can be attributed to the presence of histidine derived from rat tissues. When the number of mutants plated is small, even a slight amount of histidine allows the his\(^-\) colonies of strain TA1538 to grow and thus to eliminate the usefulness of the histidine requirement as a means of distinguishing the his\(^+\) revertants from the his\(^-\) mutants.

**Association of other bacteria**

The association of the germfree rat with salmonella strain TA1538 has also been maintained in the presence of an association with other bacteria characteristic of the intestinal microflora. In four salmonella-associated rats, Lactobacillus plantarum, Streptococcus faecalis, and Bacteroides fragilis ss vulgatus were also associated. In these rats the salmonella was maintained at the levels shown in Table 2, while each of the other three bacterial strains was found at levels higher than 10\(^6\) per g in cecal and colonic contents and in feces.

**DISCUSSION**

The maintenance of salmonella mutants in juxtaposition to the colonic mucosa provides a site-specific animal model that may be suitable for testing the correlation between carcinogenesis in animals and mutagenesis in the tester strains developed by Ames. With this model it is possible to exam-

**Table 1.** Comparison of revertant response in feces of TA1538 associated rat with that in the pour plate assay

<table>
<thead>
<tr>
<th>Compound</th>
<th>Dose/day (mg); no. of days</th>
<th>Revertant response</th>
<th>In vitro assay†</th>
<th>Liver + Liver</th>
</tr>
</thead>
<tbody>
<tr>
<td>3,2'-Dimethyl-4-aminobiphenyl</td>
<td>4; 2</td>
<td>5/8/6</td>
<td>800</td>
<td></td>
</tr>
<tr>
<td>2-Nitrofluorene</td>
<td>3.4; 3</td>
<td>8/8/2200</td>
<td>2200</td>
<td></td>
</tr>
<tr>
<td>2-Acetylaminofluorene</td>
<td>3.6; 5</td>
<td>8/8/10</td>
<td>3200</td>
<td></td>
</tr>
<tr>
<td>Azoxymethane</td>
<td>3.2; 1</td>
<td>2/5*</td>
<td>17/30</td>
<td></td>
</tr>
<tr>
<td>4-Nitrobenzene</td>
<td>50; 3</td>
<td>6/6/90</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>7-Hydroxy-2-acetylaminofluorene</td>
<td>7.5/0 6</td>
<td>21/220</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4-Nitrobenzoic acid</td>
<td>25.4</td>
<td>0/4/11</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td>Acetanilide</td>
<td>10.5</td>
<td>0/6/7</td>
<td>18</td>
<td></td>
</tr>
</tbody>
</table>

* Data are expressed as the number of rats showing an elevation in the quantity of revertants in the feces (as defined in the text) in the numerator and the number of rats tested (denominator).
† For azoxymethane, a positive response was scored for a revertant count over 1000/g for 1 day only.
‡ Compounds were tested in the pour plate assay systems with and without liver extract as previously described (7, 10). Data are expressed as revertant colonies per plate.

**Table 2.** Number of salmonella at various sites in the gastrointestinal tract

<table>
<thead>
<tr>
<th>Location</th>
<th>Salmonella count (log(_{10})/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>Forestomach</td>
<td>7.4 (4.5-8.0)*</td>
</tr>
<tr>
<td>Stomach</td>
<td>2.5 (0.0-3.7)</td>
</tr>
<tr>
<td>Duodenum</td>
<td>2.5 (1.6-4.0)</td>
</tr>
<tr>
<td>Small intestine I</td>
<td>3.8 (3.0-4.7)</td>
</tr>
<tr>
<td>Small intestine II</td>
<td>4.4 (3.6-7.2)</td>
</tr>
<tr>
<td>Small intestine III</td>
<td>5.8 (5.0-7.1)</td>
</tr>
<tr>
<td>Cecum</td>
<td>8.2 (8.0-8.6)</td>
</tr>
<tr>
<td>Colon</td>
<td>8.4 (8.2-8.6)</td>
</tr>
<tr>
<td>Feces</td>
<td>8.9 (8.3-9.3)</td>
</tr>
</tbody>
</table>

* Numbers are the median value of the colony count per g of sample followed by the range in parentheses for five rats in the control group and three treated for 3 days with 2-nitrofluorene (3.4 mg/day) prior to sacrifice.
ine whether carcinogenesis and mutagenesis, both presum-
ably related to the level of the activated chemical agent ex-
isting within the colon, can be brought about simultaneously 
by the same compounds. Thus one might determine whether 
the mutant-associated rat develops tumors after prolonged 
feeding of the test compound under conditions where a re-
vertant response occurs after only short exposure to the 
compound. For this purpose studies are currently being conduct-
ed to determine whether dose-response relationships in this 
model can be developed for various carcinogens.

In addition, the model can be extended to incorporate 
other bacteria characteristic of the intestinal microflora to 
determine whether bacterial metabolism of certain carcino-
gens (11, 23) will influence the mutagenic response in the 
tester strains. Thus the rat with salmonella associated within 
its gastrointestinal tract provides an environment for the tes-
ter strains that can be selectively modified to reflect the in-
fluence of various components of the flora as well as the in-
fluence of mammalian enzymes. A model that incorporates 
these factors may be useful in extending the value of the use 
of mutagenesis in these tester strains as an indication of car-
cinogenesis.

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