Protective Effect of Oral Salmonella enteritidis 11RX Infection against Colon Tumor Induction by 1,2-Dimethylhydrazine in Mice

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ABSTRACT

Infection of mice with Salmonella enteritidis 11RX has been shown previously to cause nonspecific immune stimulation and, consequently, resistance to subsequent challenge with a variety of transplantable tumors. The present study has examined the effect of infection with this organism in a chemical carcinogenesis system. Colonic tumors were induced in LACA and BALB/c × C57BL/6J F1 mice by weekly s.c. injection of 1,2-dimethylhydrazine (15 mg/kg) for 28 weeks. Infection of mice p.o. with live S. enteritidis 11RX at 8-week intervals during 1,2-dimethylhydrazine administration protected both strains against colon tumorigenesis. Significantly fewer infected than control BALB/c × C57BL/6J F1 mice had colonic tumors at or before termination of the experiment (34 or 40 weeks) (p < 0.001 in all cases). Comparable results were obtained with both male and female mice. The difference in tumor incidence between control and infected LACA mice was not statistically significant, however; the number and size of the lesions was greater in control mice (p < 0.02). Although it has not been proven that the protective effect is mediated by the immune system, the results are consistent with the operation of a macrophage-mediated surveillance system.

It is suggested that enteric infections should be considered as a possible contributing factor in the epidemiology of human colonic cancer.

INTRODUCTION

Induction of colonic tumors in mice and rats by DMH is widely used as an experimental model for studies of the role of environmental factors in colon carcinogenesis in humans (e.g., Refs. 17 and 26). We have used this model system to investigate the effect of enteric infection on colonic tumor induction in mice.

Salmonella species are natural enteric pathogens. The organism used in this study, Salmonella enteritidis 11RX, is a rough strain of low virulence. Infection of mice with this organism i.v., i.p., or p.o. leads to the establishment of a carrier state with controlled growth of bacteria in the liver and spleen. As with certain other intracellular microbial parasites (cf. Ref. 21), the infection elicits a sequence of immunological events (5, 13, 19) leading to the production of activated macrophages which are nonspecifically cytotoxic for tumor cells in vivo and in vitro (3). After the carrier state has ended (about 40 days after infection) and resistance to tumor challenge has declined, the latter can be “recalled” by injection of 11RX protein antigen at the site of tumor challenge (4, 6).

Activated macrophages have been suggested (e.g., Ref. 1) to have a function in immune surveillance against neoplasia. Since S. enteritidis combines the properties of a natural enteric pathogen and the ability to bring about macrophage activation, it seemed a particularly suitable organism to test for a protective effect against colon tumor induction. The experiments reported here demonstrate that repeated p.o. challenge with live S. enteritidis 11RX during the course of DMH administration significantly and reproducibly reduced the incidence and/or extent of colonic tumors in 2 strains of mice.

MATERIALS AND METHODS

Mice. BALB/c × C57BL/6J F1 mice were bred from breeding pairs obtained from The Jackson Laboratory, Bar Harbor, Maine. LACA mice are bred as a closed colony from stock obtained approximately 10 generations ago from the Medical Research Council, Carshalton, United Kingdom. Mice were 8 to 10 weeks old at the beginning of the experiments and 12 to 16 weeks old at the time of initial DMH administration.

Treatment. DMH (K & K Fine Chemicals, Cleveland, Ohio; Lot 15009-A) solutions, freshly prepared prior to injection by dissolving in 1 mM EDTA and adjusting to pH 6.5 with NaHCO3 were injected s.c. (at a dose of 15 mg/kg) at 1-week intervals. Animals were weighed at monthly intervals, and the dose was adjusted in proportion to any change in weight. In both infected and control groups, the average weight of mice was always in the range 20 to 22 g for female mice and 23 to 25 g for male mice throughout DMH administration. Details of the duration of DMH administration for each experiment are given in “Results.”

Bacterial Infection. Where indicated, mice were immunized with S. enteritidis by i.v. injection of 105 live organisms (5 × 10–2 units of the dose that kills 50% of animals) from a log-phase culture 4 to 8 weeks prior to commencement of DMH administration. This immunization prevented morbidity due to the bacterial infection during carcinogenesis. Infection p.o. was effected by introduction of 106 or 1010 bacteria (as indicated) in 0.5 ml 0.9% NaCl solution into the esophagus using a smooth-ended 19-gauge needle. For the study of persistence of organisms, a streptomycin-resistant mutant of S. enteritidis 11RX was isolated. The course of infection of mice by this mutant was identical with that of the parental strain. The liver, spleen, intestine were excised from groups of 3 mice at various times after infection, homogenized with an Ultra-Turrax in 0.9% NaCl solution, and aliquots were plated on nutrient agar containing streptomycin (100 μg/ml). S. enteritidis colo-
nies were readily distinguishable from the few other streptomycin-resistant colonies in the intestinal isolates by its characteristic rough colony morphology.

Scoring of Results. Mice were checked twice weekly for blood in the feces as an indicator of the presence of tumor. Any mice with blood in the feces were killed and autopsied. At termination of the experiment, all the mice were killed and autopsied. The colon was removed and opened, and tumors, where present, were scored according to the method of Evans et al. (15).

Histology. This phase of the study was carried out in blind fashion on coded, randomized specimens. All the colons, and in selected cases the liver, were fixed in 10% formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin for microscopic examination.

RESULTS

Persistence of S. enteritidis 11RX after Challenge p.o. The dose of bacteria administered p.o. in these experiments (10^10 organisms for BALB/c x C57BL/6J F1, mice; 10^9 organisms for LACA mice) was sufficient to allow penetration of the gut and allow establishment of a carrier state with growth of bacteria in the liver and spleen of previously unimmunized mice. Administration p.o. of the same dose of bacteria to mice which had been immunized with 10^7 11RX i.v. 28 days previously was followed by rapid clearance of organisms from the liver and spleen, with few or no bacteria recovered after 2 days. However, bacteria persisted in the gut at detectable levels (>10^3) for 9 to 12 days (compared with >24 days in the case of primary p.o. infection).

Tumor Induction by DMH. It has been reported previously that different strains of mice vary greatly in their susceptibility to DMH carcinogenesis (11, 14, 15). The LACA mice used in this study are highly susceptible to carcinogenesis in this and another (7) system, whereas the BALB/c x C57BL/6J F1 mice are relatively resistant in both systems. When mice of both strains were treated with DMH, as described in "Materials and Methods," for 28 weeks with a follow-up period of 12 weeks, 22 of 31 LACA mice compared with 20 of 48 BALB/c x C57BL/6J F1 mice developed colonic tumors. The difference in incidence was statistically significant (\( \chi^2 = 5.37; p < 0.05 \)) and was borne out by subsequent experiments (cf. Tables 2 and 3).

DMH carcinogenesis was highly organ specific. Of all 359 mice examined, 245 had colonic tumors, 6 had liver tumors, and 3 had urogenital tumors.

Effect of p.o. infection with S. enteritidis 11RX on Colon Carcinogenesis by DMH. Results of 2 separate experiments are shown in Tables 1 and 2. In each case BALB/c x C57BL/6J F1 mice had a significantly lower incidence of colonic tumors when they were subjected to repeated infection with S. enteritidis 11RX (\( p < 0.001 \)). This was true for both male and female mice (cf. Table 2). The difference in incidence between male and female mice was not significant.

The difference in tumor incidence between control and infected LACA mice was not statistically significant (perhaps because the experiment was allowed to progress too far). However, when the tumors were macroscopically scored according to the method of Evans et al. (15), a significant difference was observed in the number and size of tumors (Table 3). Similarly, tumor progression was more advanced in control than in S. enteritidis 11RX-infected BALB/c x C57BL/6J F1 mice (Table 3).

Histopathology. In the whole series, tumors observed ranged from microscopic adenomas to invasive adenocarcinomas. The histology of the lesions was similar to that described by others (17). The stage of invasion of the adenocarcinomas observed ranged from carcinoma in situ to complete penetration of the muscularis propria of the colorectum. However, in the majority the invasion was confined to the submucosa. Most of the adenocarcinomas were well differentiated (45%) or moderately differentiated (48%), whereas few were poorly differentiated (7%).

Although on macroscopic scoring, control LACA mice (Table 3) had more and larger tumors than did the infected group, there was no significant difference between the 2 groups in the proportion of mice which had adenocarcinomas in addition to adenomas. However, the total number of adenocarcinomas observed was greater in the control group than in the infected group. Eighteen of the 30 control mice had a total of 33 adenocarcinomas, and 16 of the 30 infected mice had a total of 24 adenocarcinomas.

In the case of the BALB/c x C57BL/6J F1, mice (Table 3, Experiment 2), 2 of the 8 control mice and 10 of the 26 infected mice, scored macroscopically as tumor negative, showed microscopic adenomas. Taking this into account, the proportion of mice macroscopically and microscopically tumor free was still significantly higher in infected mice (16 of 54) than in control mice (6 of 69) (\( \chi^2 = 7.70; p < 0.01 \)). Most of the tumor-positive mice in both groups had only benign adenomas; however, 16 of 69 control mice and 3 of 54 infected mice had adenocarcinomas as well as adenomas at the final scoring. The difference in the proportion of mice with adenocarcinomas in the 2 groups was statistically significant (\( \chi^2 = 5.92; p < 0.02 \)). The control group of 69 mice had a total of 166 adenomas and 16 adenocarcinomas, whereas the infected group of 54 mice had a total of 62 adenomas and 3 adenocarcinomas. The ratio of adenocarcinomas to adenomas was not significantly different between the 2 groups (\( \chi^2 = 0.66; p = 0.6 \)). This implies that, although infection reduced the number of tumors induced and/or increased their latency period, it did not affect the progression of tumors from benign to malignant.

<table>
<thead>
<tr>
<th>Group</th>
<th>Original no.</th>
<th>Survivors at 28 wk</th>
<th>Survivors at 40 wk</th>
<th>Colonic tumors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>54</td>
<td>49</td>
<td>48</td>
<td>20</td>
</tr>
<tr>
<td>Infected</td>
<td>45</td>
<td>31</td>
<td>31</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 1

Effect of p.o. infection with S. enteritidis 11RX on colonic tumor incidence (Experiment 1)

DMH was administered to BALB/c x C57BL/6J F1, mice, as described in "Materials and Methods," for 28 weeks. Twelve weeks later, mice were killed and autopsied. Infected mice were immunized with 10^10 live S. enteritidis 11RX i.v. 8 weeks prior to the beginning of the experiment, and were challenged p.o. with 10^10 live organisms after 8, 16, and 24 weeks of DMH injection. The incidence of colonic tumors was significantly higher in the control group than in the infected group (\( \chi^2 = 24.2; p < 0.001 \)).

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Table 2

Effect of p.o. infection with S. enteritidis 11RX on colonic tumor incidence (Experiment 2)

DMH was administered as described in "Materials and Methods." LACA mice received weekly injections for 28 weeks and were killed at 30 weeks. BALB/c x C57BL/6J F1 mice received weekly injections for 32 weeks and were killed at 34 weeks. Infected mice were immunized with 10^9 live S. enteritidis 11RX i.v. 4 weeks prior to the first DMH injection and received live S. enteritidis p.o. at 8-week intervals during DMH administration (4 doses of 10^9 organisms for F1 mice; 3 doses of 10^8 organisms for LACA mice).

<table>
<thead>
<tr>
<th>Group</th>
<th>Original no.</th>
<th>No. of mice with tumors before final day</th>
<th>Survivors to final day</th>
<th>No. with colonic tumors at final scoring</th>
<th>Total no. of mice</th>
<th>No. of mice tumor free</th>
<th>$\chi^2$</th>
<th>df</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>LACA female (control)</td>
<td>48</td>
<td>1</td>
<td>30</td>
<td>30</td>
<td>31</td>
<td>0</td>
<td>1.95</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>LACA female (infected)</td>
<td>44</td>
<td>6</td>
<td>30</td>
<td>26</td>
<td>32</td>
<td>4</td>
<td>0.2</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>BALB/c x C57BL/6J F, male (control)</td>
<td>50</td>
<td>17</td>
<td>29</td>
<td>22</td>
<td>39</td>
<td>7^b</td>
<td>12.58</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>BALB/c x C57BL/6J F, male (infected)</td>
<td>50</td>
<td>1</td>
<td>29</td>
<td>12</td>
<td>13</td>
<td>17^c</td>
<td>0.001</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>BALB/c x C57BL/6J F, female (control)</td>
<td>50</td>
<td>7</td>
<td>40</td>
<td>39</td>
<td>46</td>
<td>1^b</td>
<td>23.14</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>BALB/c x C57BL/6J F, female (infected)</td>
<td>50</td>
<td>3</td>
<td>25</td>
<td>16</td>
<td>19</td>
<td>9^c</td>
<td>0.001</td>
<td>1</td>
<td>0</td>
</tr>
</tbody>
</table>

Extent of colorectal carcinogenesis in BALB/c x C57BL/6J F1 and LACA mice

Experimental details were as described in Table 2. Tumors were classified macroscopically as described by Evans et al. (15). The difference in tumor stage between control and infected BALB/c x C57BL/6J F1 mice was statistically significant ($\chi^2$ (3 d.f.) = 29.16; $p < 0.001$). The difference in tumor stage between control and infected LACA mice was also statistically significant ($\chi^2$ (4 d.f.) = 9.70; $p < 0.05$). The use of a 5 x 2 contingency table takes no account of the relationship between the different tumor stages. When columns were grouped as Columns 0, 1, and 2 and Columns 3 and 4 and analyzed using a 2 x 2 contingency table, the difference was significant at 98% level ($\chi^2$ (1 d.f.) = 5.63; $p < 0.02$).

Table 3

Extent of colorectal carcinogenesis in BALB/c x C57BL/6J F1 and LACA mice

<table>
<thead>
<tr>
<th>Tumor stage</th>
<th>0</th>
<th>½</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>BALB/c x C57BL/6J F, mice</td>
<td>8</td>
<td>14</td>
<td>33</td>
<td>14</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control mice (males + females)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Infected mice (males + females)</td>
<td>26</td>
<td>15</td>
<td>12</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LACA mice</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control mice</td>
<td>0</td>
<td>5</td>
<td>8</td>
<td>8</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>Infected mice</td>
<td>4</td>
<td>9</td>
<td>10</td>
<td>4</td>
<td>3</td>
<td></td>
</tr>
</tbody>
</table>

DISCUSSION

The potential effects of microorganisms on carcinogenesis in experimental animals have been recognized for some time (18). Recently, attention has been focused on the possible role of microflora in carcinogen and/or promoter metabolism, particularly in the case of colon carcinoma (e.g., Refs. 16, 20, and 23). Reddy et al. (24) found germ-free rats less susceptible to DMH carcinogenesis than were conventional rats; however, the converse was true in the case of azoxymethane, which is an intermediate in the metabolic activation of DMH (28). Although certain bacterial infections elicit immune responses which are effective in inhibiting tumor growth (e.g., Ref. 8), little consideration has been given to the possible role of enteric pathogens (or, indeed, the normal bowel flora) as immune stimulants and antagonists of colon cancer development. BCG, which elicits well-documented antitumor activity in other systems (8), is one of the few microorganisms which have been tested in the colon carcinogenesis model. Rogers and Gilden (25) administered live BCG i.c. to rats treated with DMH, but did not observe any inhibition of tumorigenesis. Recently Cruse et al. (12) reported that i.p. administration of killed Corynebacterium parvum enhanced DMH carcinogenesis in rats.

Systemic infection of mice with S. enteritidis 11RX has been previously shown in this laboratory to lead to the development of strong cell-mediated antibacterial immunity and, concomitantly, reticuloendothelial system stimulation with the production of activated cells, presumably macrophages, which are capable of killing tumor cells in vivo and in vitro (3–6, 13, 19). Since Salmonella species are natural enteric pathogens, it was of interest to investigate whether p.o. infection with S. enteritidis 11RX could protect mice against colonic tumor induction by DMH. Any organisms which may penetrate the gut after p.o. administration to immune mice are rapidly cleared. This means that the regimen used in this study is probably equivalent to repeated local immune recall in the gut. The use of live organisms avoids problems of antigen degradation which would occur if killed preparations were used, and it ensures longer persistence in the gut.

In these experiments, we consistently found a reduced extent of tumorigenesis in mice repeatedly infected p.o. with S. enteritidis 11RX as compared with control mice. The protective effect was greater in BALB/c x C57BL/6J F1 mice than in LACA mice. Similarly, BALB/c x C57BL/6J F1 mice are more effectively protected against the growth of Ehrlich ascites tumor by S. enteritidis 11RX infection than are LACA mice. This

* I. Kofarski, L. K. Ashman, and M. P. Ashley, unpublished observations.
appears to parallel lower resistance to Salmonella infection and a weaker cellular immune response to bacterial antigens in LACA mice. The reason why S. enteritidis 11RX protected against DMH colon carcinogenesis, whereas other workers found that BCG and C. parvum, which also activate macrophages, were not protective (in rats), is not known. However, it is noteworthy that both BCG (10, 22) and C. parvum (9) have been reported to enhance tumor growth in certain circumstances. In contrast, inhibition but no enhancement of tumor growth has been observed using S. enteritidis 11RX against 6 different transplantable tumors in 3 strains of mice (6, 27).

Our experiments do not permit distinction between an effect of S. enteritidis 11RX infection on numbers of tumor foci induced and its effect on an increase in the latency period of tumors after induction. Nor can we conclude that the protective mechanism is necessarily immunological. Nevertheless, the results are consistent with the existence of a surveillance system mediated by activated macrophages. Furthermore, the results suggest that epidemiological studies of the possible modulating influence of enteric infection on the incidence of colon cancer in human populations are warranted. It is well known that there are wide geographical differences in colon cancer incidence and that these are related to parameters such as gross national product which reflect the "standard of living" (2). These differences are frequently attributed to differences in dietary composition. It may be that high standards of hygiene, and consequently a low frequency of enteric infections, also contribute to the high incidence of colon cancer in developed Western countries.

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REFERENCES

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