ABSTRACT Red meat intake is associated with colon cancer risk. Puzzlingly, meat does not promote carcinogenesis in rat studies. However, we demonstrated previously that dietary heme promotes aberrant crypt foci (ACF) formation in rats given a low-calcium diet. Here, we tested the hypothesis that heme-rich meats promote colon carcinogenesis in rats treated with azoxymethane and fed low-calcium diets (0.8 g/kg). Three meat-based diets were formulated to contain varying concentrations of heme by the addition of raw chicken (low heme), beef (medium heme), or black pudding (blood sausage; high heme). The no-heme control diet was supplemented with ferric citrate and the heme control diet with hemoglobin to match iron and heme concentrations in the beef diet, respectively. After 100 d, colons were scored for ACF and mucin-depleted foci (MDF). Fecal water was assayed for lipoperoxides and cytotoxicity. Only diets with heme promoted the formation of MDF, but all meat diets promoted ACF formation. The number of MDF/colon was 0.55 ± 0.08 in controls, but 1.2 ± 0.6 (P = 0.13), 1.9 ± 1.4 (P < 0.01), and 3.0 ± 1.2 (P < 0.001) in chicken-, beef-, and black pudding–fed rats. MDF promotion by the high-heme black pudding diet was greater than that by the medium-heme beef diet. The number of ACF/colon was 72 ± 16 in controls, but 91 ± 18, 100 ± 13, and 103 ± 14 in chicken-, beef-, and black pudding–fed rats (all P < 0.001). ACF and MDF did not differ between rats fed the beef diet and those fed the heme control diet. MDF promotion was correlated with high fecal water lipoperoxides and cytotoxicity (r = 0.65, P < 0.01). This is the first study to show the promotion of experimental carcinogenesis by dietary meat and the association with heme intake. J. Nutr. 134: 2711–2716, 2004.

KEY WORDS: • colorectal carcinogenesis • heme • lipoperoxidation • red meat • chicken

Colorectal cancer is a major cause of death in affluent countries, and recommendations are to reduce red meat intake to reduce the risk (1). A meta-analysis of epidemiological studies by Norat et al. (2) found a moderate but significant association between red meat intake and colorectal cancer risk. In puzzling contrast with epidemiological studies, experimental studies do not support the hypothesis that red meat increases colorectal cancer risk. Among the 12 rodent studies reported in the literature, none demonstrated a specific promotional effect of red meat (3–14). McIntosh et al. (3) showed that rats given a diet containing kangaroo meat, soybean protein, or casein have a similar incidence of dimethylhydrazine-induced tumors. Clinton et al. (4) also found the colon tumor incidence to be the same for beef meat–(raw or grilled) and soybean diet–fed rats.


2 The study was supported by the INRA, the DGER, and the French region Midi-Pyrénées.

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(ACF) formation compared to casein-fed controls. Belobrajdic et al. (14) found kangaroo meat to promote aberrant crypt foci (ACF)\textsuperscript{4} formation in comparison with whey protein, but whey is known to protect against colon carcinogenesis (15).

Sesink et al. (16) speculated that heme, found in red meat myoglobin, would enhance colon carcinogenesis. They demonstrated that pure hemin added to rats diet increases colonic epithelial proliferation and that calcium phosphate inhibits the hemin-induced proliferation (17). In line with Sesink’s hypothesis, we showed that heme diets increase the number and size of azoxymethane-induced ACF in rats fed a low-calcium diet, while hemoglobin diets increase ACF number only (18). Dietary hemin also produces cytotoxic fecal water and high amounts of TBARS, indicative of lumen lipoperoxidation (16), while dietary hemoglobin increases fecal TBARS only (18). ACF are putative preneoplastic lesions, and the effect of agents on ACF is correlated with the effect on tumor incidence. They were distributed randomly in pairs into stainless-steel wire-bottom cages. The room was kept at a temperature of 22°C on a 12-h light-dark cycle. Rats were allowed 7 d of acclimatization to the room and to the control diet (Table 1) before being injected i.p. with the carcinogen azoxymethane (Sigma Chemical, 20 mg/kg body wt) in NaCl (9 g/L). Seven days after the injection the rats were allowed free access to their respective diets for 100 d. Feed was changed every 2 or 3 d and water once a week. Body weights were measured at the indicated time points. ABCC (18) reported that the black pudding diet could not be balanced for iron (0.95 g/kg). The diets were prepared twice a month and maintained at −20°C. A TBARS assay showed no lipoperoxidation (data not shown).

**Materials and Methods**

Animals. Sixty Fisher 344 female rats were purchased at 4 wk of age from Iffa Credo. Animal care was in accordance with the guidelines of the European Council on animals used in experimental studies. They were distributed randomly in pairs into stainless-steel wire-bottom cages. The room was kept at a temperature of 22°C on a 12-h light-dark cycle. Rats were allowed 7 d of acclimatization to the room and to the control diet (Table 1) before being injected i.p. with the carcinogen azoxymethane (Sigma Chemical, 20 mg/kg body wt) in NaCl (9 g/L). Seven days after the injection the rats were allowed free access to their respective diets for 100 d. Feed was changed every 2 or 3 d and water once a week. Body weights were monitored weekly. Feed intake per cage of 2 rats was also monitored.

Diet. Experimental diets, as shown in Table 1, were based on AIN-76 diet (22) prepared and formulated in a powdered form by the UPAE (INRA). Dibasic calcium phosphate was included at a low concentration of 2.7 g/kg. Three meat diets given to 3 groups of rats (n = 10 rats/group) were formulated to contain varying concentrations of heme as hemoglobin or myoglobin by the addition of freeze-dried beef, chicken, or black pudding at 600 g/kg meat of the total diet. The beef and chicken (skinless) meat was obtained from UPAE. The beef contained 0.6 μmol/g of heme while none was detected in the chicken diet (see the assay below). The low fat black pudding (blood sausage) contained 1527 mg/kg heme. Other nutrients were balanced: 50% protein, 20% fat, 18% carbohydrate, and 0.8 g/kg calcium (based on added components, no analysis was done on whole diets).

![Table 1](https://www.nutrition.org/content/122/12/2712.full.html)

**Table 1.** Composition of diets.

<table>
<thead>
<tr>
<th>Control</th>
<th>Chicken</th>
<th>Beef</th>
<th>Black pudding</th>
<th>Hemoglobin</th>
</tr>
</thead>
<tbody>
<tr>
<td>g/kg</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chicken</td>
<td>0</td>
<td>0</td>
<td>600</td>
<td>0</td>
</tr>
<tr>
<td>Beef</td>
<td>0</td>
<td>0</td>
<td>600</td>
<td>0</td>
</tr>
<tr>
<td>Black pudding</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>600</td>
</tr>
<tr>
<td>Hemoglobin</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

1. Low-calcium casein.
2. AIN-76 mix, but 500 g/kg of dibasic calcium phosphate replaced by sucrose in mineral mix.
3. All diets contained 140 mg/kg iron except the black pudding diet (850 mg/kg). Iron concentration was measured in freeze-dried meat before the diets were prepared: chicken: 37.5, beef: 172.6, and black pudding 1527 mg/kg. Other nutrients were balanced: 50% protein, 20% fat, 18–25% carbohydrate, and 0.8 g/kg calcium (based on added components, no analysis was done on whole diets).

4 Abbreviations used: ACF, aberrant crypt foci; HAB-AB, high-iron diamine-Alcian blue procedure; MDF, mucin-depleted foci; MTT, 3-(4,5-dimethylthiazol-2-y)-2,5 diphenyl tetrazolium bromide.

Cytolytic assay of fecal water. The cytotoxicity of fecal water was quantified by 2 methods, on erythrocytes and on a cell line. First,
the cytolytic activity of fecal water was quantified by potassium release from erythrocytes as described by Govers et al. (26). Second, the cytotoxicity of fecal water obtained with a different method (see above) was also quantified by the 3-(4,5-dimethyldiazol-2-yl)-2,5 diphenyl tetrazolium bromide (MTT) test on a cell line according to Bonneson et al. (27). Briefly, the cancerous mouse colonic epithelial cell line, CMT93 (ECAC), was seeded in 96-well microtiter plates (1.6 × 10^5 cells per well in 200 μL of medium) and at confluence the cells were treated for 24 h with the fecal water sample to be tested and diluted in the culture medium at a concentration of 10% (v:v). Each fecal water sample was tested in 7 wells and 10 wells remained untreated to act as controls. One hundred micromolars of MTT (9% in PBS) was added to each well. After 3 h of incubation at 37°C in the dark, 100 μL of a 10% SDS-0.1 mol/L NaOH mixture was added. After 1 h of incubation in the dark, the absorbance of each well was read using a microplate reader at wavelength 570 nm for cytotoxicity and 690 nm for background.

Statistical analysis. Results were analyzed using Systat 10 software for Windows and reported as means ± SD. ACF scoring was done in duplicate. ACF variables were tested first using 2-way (groups and readers) ANOVA. The (group × reader) interaction was never significant, and when total ANOVA was significant (P < 0.05), pairwise differences between groups were analyzed using Fisher's least-significant-difference test. MDF variables and all other data were analyzed using 1-way ANOVA and groups were compared using Bonferroni correction for multiple comparisons. Because the black pudding diet contained a very high concentration of heme, heme values were log-transformed before statistical analysis.

RESULTS

Weight gain and feed intake. Beef-fed rats quickly became heavier than control rats, and the difference was significant at d 30. The final body weight of beef-fed rats was greater than that of controls (P < 0.05, Table 2). Black pudding–fed rats had wetter stools, a known effect of dietary heme, and they drank more water than controls (22 ± 1 mL/d vs. 16 ± 0.5 mL/d, P < 0.001). Furthermore, all groups had similar food intakes; at day 75, intake was 8.4 ± 0.5 g/d (full data not shown).

ACF. All meat-based diets (chicken, beef, and black pudding) increased the number of ACF (P < 0.001, Fig. 2A) and the number of aberrant crypts per colon (P < 0.001, Table 2) after 100 d. Chicken and black pudding, but not beef, also increased the number of crypts per ACF (P < 0.01, Table 2). Aberrant crypts and ACF promotion by the black pudding diet were more potent than promotion by the chicken diet (P < 0.05, Table 2). Rats fed the beef diet did not differ from those fed the hemoglobin diet in aberrant crypt number or ACF per colon. However, the ACF contained more crypts in the hemoglobin-fed group (Table 2).

MDF. Beef- and black pudding–fed rats had more MDF than control rats (P < 0.01), and promotion by black pudding was more potent than promotion by beef (P < 0.05, Fig. 2B). The chicken-based diet, the low-heme diet, did not promote MDF formation (Fig. 2B). The effects on the number of MDF also occurred on the number of mucin-depleted crypts (Table 2). The groups did not differ in the number of crypts per MDF. The beef and hemoglobin groups did not differ for any of the variables tested (Table 2).

Fecal heme, TBARS, and cytotoxicity. The fecal concentration of heme matched the heme intake. As expected, no heme was detected in feces of control and chicken diet–fed rats (Table 3). The analysis of fecal samples stored during the study of Parnaud et al. (13) where diet containing 60% beef meat but 130 μmol/g calcium yielded similar results: No heme was detected in feces of control and chicken diet–fed rats, but there was 1.7 ± 1.5 μmol/g in feces of beef-fed rats. However, in the present study, the heme concentration was higher in the feces of hemoglobin-fed rats than in beef-fed rats (Table 3). This is consistent with the observation that less heme iron reaches the colon when it is supplied as red meat rather than in hemoglobin form (14). We measured the characteristics of fecal water because, according to studies on bile acids, the soluble fraction of colonic contents would interact more strongly with the mucosa than the insoluble fraction (28). As expected, the heme concentration in fecal water depended directly on the level of heme in the diet (Table 3), with, as noted above, a difference between meat- and hemoglobin-fed rats. There was no heme in fecal waters in Parnaud's meat study, even in samples from rats given a 60% beef diet (13).

Heme can induce the formation of peroxyl radicals in fats, which may be cytotoxic and cleave DNA in vivo (29). Lipid

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Effect of meat-based diets on ACF and MDF formation in the colon of rats 107 d after the injection of azoxymethane1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diets</td>
<td>Heme (μmol/g)</td>
</tr>
<tr>
<td>---------</td>
<td>--------------</td>
</tr>
<tr>
<td>Control</td>
<td>0.0</td>
</tr>
<tr>
<td>Chicken</td>
<td>0.0</td>
</tr>
<tr>
<td>Beef</td>
<td>0.36</td>
</tr>
<tr>
<td>Hemoglobin</td>
<td>0.36</td>
</tr>
<tr>
<td>Black pudding</td>
<td>9.54</td>
</tr>
</tbody>
</table>

1 Values are means ± SD. Means in columns with superscripts without a common letter in differ, P < 0.05.
The fecal water of hemin-fed rats is cytotoxic, which would explain the hemin-induced increased proliferation (18). Cytotoxicity of fecal water was measured by 2 methods: lysis of erythrocytes and toxicity on CMT93 cells. The black pudding diet and beef diet increased TBARS by 2- to 4-fold (all increased TBARS in the fecal water by 23-fold. The heme concentration was higher in the diet (Table 3). Taken together, these data suggest that cytotoxicity, pH, and lipoperoxides of fecal water are associated with heme intake and fecal heme. Indeed, significant correlations were seen between heme intake and fecal water cytotoxicity ($r = 0.98$, pH ($r = 0.86$), and TBARS ($r = 0.73$), all $P < 0.01$, $n = 30$ cages of 2 rats).

**DISCUSSION**

This study is the first to show that meat can specifically promote colon carcinogenesis. In addition, the promoting effect was stronger than other promoting agents (30) and clearly associated with the heme concentration in meat. This study was done with a low-calcium diet containing 60% meat and 5% easily oxidized oil. We used 2 putative precancerous endpoints: the established ACF and the recently described MDF. Heme in the diet led to ACF and MDF promotion in the colon. The low-heme chicken-based diet did not promote MDF, but increased the ACF number.

This study is, to our knowledge, the first non-Italian study to use a new carcinogenesis endpoint which was recently described by Caderni et al. (20). MDF may predict tumor outcome better than ACF, as shown in the studies of symbiotics, cholic acid, and piroxicam (20,21). We found that MDF were quite easy to score, but we detected fewer MDF per control rat than did Caderni et al. (20). This is likely the result of the carcinogen dose: azoxymethane was injected once instead of twice, and the resulting number of ACF was 75% fewer here than in the study of Caderni et al. (72 vs. 298 ACF/colon).

That heme content in meat was responsible for promotion of colon carcinogenesis, at least in part, is supported by the following facts: (i) all tested meat diets promoted ACF formation, but this was significantly greater in rats fed the high-heme diet, based on black pudding, than for those fed the low-heme chicken diet (Fig. 2). (ii) Only heme-containing diets promoted MDF formation, and the effect was dose dependent, because the black pudding effect was significantly stronger than the beef effect. MDF per colon was correlated with heme intake ($r = 0.63$, $n = 60$, $P < 0.01$). (iii) Beef and hemoglobin diets, which provided the same amount of heme, produced equivalent effects: no lytic activity on erythrocytes, but a 4-fold increase in CMT93 cell toxicity ($P < 0.001$). The cytotoxicity of fecal water from chicken-fed rats was not different from that of controls (Table 3). All meat-based diets increased the fecal pH, which was higher when the heme concentration was higher in the diet (Table 3). Taken together, these data suggest that cytotoxicity, pH, and lipoperoxides of fecal water are associated with heme intake and fecal heme.

**TABLE 3**

<table>
<thead>
<tr>
<th>Diet</th>
<th>Heme intake$^2$</th>
<th>Dry fecal mass</th>
<th>Heme in feces$^2$</th>
<th>Heme in fecal water$^2$</th>
<th>TBARS in fecal water, MDA equivalents</th>
<th>pH of fecal water</th>
<th>Cytolytic activity on erythrocytes</th>
<th>Cytotoxicity on CMT93 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\mu$mol/d</td>
<td>g/d</td>
<td>$\mu$mol/g</td>
<td>$\mu$mol/L</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>$0^a$</td>
<td>$0.50 \pm 0.11^a$</td>
<td>$0^a$</td>
<td>$0^a$</td>
<td>$40 \pm 15^a$</td>
<td>$7.85 \pm 0.03^a$</td>
<td>$1 \pm 2^a$</td>
<td>$12 \pm 12^a$</td>
</tr>
<tr>
<td>Chicken</td>
<td>$0^a$</td>
<td>$0.58 \pm 0.06^b$</td>
<td>$0^a$</td>
<td>$0^a$</td>
<td>$69 \pm 16^a$</td>
<td>$8.02 \pm 0.03^b$</td>
<td>$1 \pm 2^a$</td>
<td>$26 \pm 15^a$</td>
</tr>
<tr>
<td>Beef</td>
<td>$3.0 \pm 0.4^b$</td>
<td>$0.64 \pm 0.09^b$</td>
<td>$0.5 \pm 0.2^b$</td>
<td>$19 \pm 7^b$</td>
<td>$138 \pm 17^b$</td>
<td>$8.17 \pm 0.03^c$</td>
<td>$1 \pm 2^a$</td>
<td>$59 \pm 14^b$</td>
</tr>
<tr>
<td>Hemoglobin</td>
<td>$2.9 \pm 0.4^b$</td>
<td>$0.53 \pm 0.07^b$</td>
<td>$0.9 \pm 0.3^c$</td>
<td>$52 \pm 47^c$</td>
<td>$195 \pm 96^c$</td>
<td>$8.13 \pm 0.03^c$</td>
<td>$1 \pm 1^a$</td>
<td>$58 \pm 27^b$</td>
</tr>
<tr>
<td>Black pudding</td>
<td>$87.0 \pm 8.0^c$</td>
<td>$1.00 \pm 0.06^c$</td>
<td>$23.6 \pm 8.6^d$</td>
<td>$1097 \pm 484^d$</td>
<td>$975 \pm 229^c$</td>
<td>$8.30 \pm 0.06^c$</td>
<td>$73 \pm 36^b$</td>
<td>$88 \pm 03^c$</td>
</tr>
</tbody>
</table>

1 Values are means $\pm$ SD, $n = 5$ or 10 cages (controls). Means in columns with superscripts without a common letter differ, $P < 0.05$.
2 Log-transformed data were tested by ANOVA.
promoted ACF and MDF equally (Table 3). This meat study is thus consistent with our previous study, where ACF were promoted dose-dependently by graded doses of dietary hemin (18). We think that previous studies in rats failed to show that red meat promotes carcinogenesis because meat was included in a high-calcium diet. The standard AIN-76 diet contains 130 mmol/kg calcium, which is similar to the concentration that inhibits heme-induced colonic proliferation (17) and heme-induced ACF promotion (18). Calcium precipitates heme in the gut lumen and reduces heme concentration in fecal water (17,18). In the study of Parnaud et al. (13), the heme concentration was high in the feces of beef-fed rats, but was not detectable in the fecal water (see results above). We suggest that this is due to high dietary calcium, and it resulted in the lack of ACF promotion by the beef diet (13). However, the link between heme intake and ACF yield is not a direct one; black pudding provided a huge quantity of heme to the gut that was not mirrored linearly in the ACF outcome.

The mechanism of how the heme promotes ACF formation and induce lipoperoxidation and cytotoxicity of fecal water (18). Indeed, heme promotes the nonenzymatic peroxidation of PUFA (16,18,29). The lipid peroxyl radicals (LOO•) generated from simultaneous fat and heme iron ingestion, and the resulting oxygen radicals, can cleave DNA or modify DNA bases, which could increase carcinogenesis (29). The beef-based diet contained 0.36 μmol/g heme. Its intake led to 19 μmol/L heme in fecal water and a 2.5-fold increase in lipoperoxidation (Table 3). Similar TBARS values were seen in fecal water from beef-fed rats and, in our previous study (18), from hemoglobin diet–fed rats (138 and 187 μmol/L MDA equivalents, respectively). In addition, red meat intake induced fecal cytotoxicity and increased the pH of fecal water (Table 3). Black pudding contains 25 times more heme than beef. Compared with beef, the consumption of black pudding led to 60 times more heme in fecal water, 7 times more TBARS, and a much higher cytotoxicity (Table 3). Fecal water from black pudding–fed rats strikingly induced erythrocyte cytosis. Thus, we conclude that there was a dose-dependent effect of the heme concentration in the diet and in fecal water on the fecal lipoperoxidation, cytotoxicity, and pH. All correlations among these variables were significant. In addition, MDF and ACF numbers per rat were also correlated with these fecal values (all r > 0.5, all P < 0.01, n = 60 rats, highest correlation, r = 0.65 between number of MDF and cytotoxicity). These correlations suggest that fecal cytotoxicity, lipoperoxidation, and pH are heme concentration-related features of colon carcinogenesis. That hemoglobin and meat diets, with same heme content as hemoglobin and myoglobin, produced the same effects also supports this idea (Table 3). Surprisingly, a published study with a protocol very similar to this one noted no ACF promotion (14). Fecal heme concentrations were similar in both studies, but the fecal TBARS value was 2 times higher in the study of Belobradjic et al. (14). We speculate that lipoperoxidation was inhibited by tert-butylhydroquinone in the AIN-93 diet used by Belobradjic et al. (14). This chance observation supports the hypothesis that heme-induced lipoperoxidation plays a role in the promotion of colon carcinogenesis.

The low-heme chicken-based diet surprisingly increased the ACF number and size (Table 2). The chicken meat used in this study may contain a promoter that is not heme and remains to be explained. The prominent features of the chicken diet were high arachidonic acid and niacin. The chicken diet contained 1 g/kg of arachidonic acid [calculated from (31)] compared to 0.25 g/kg in other diets. Arachidonic acid has pro-tumorigenic properties, likely by increasing prostanoid synthesis (32). In addition, the chicken diet contained 207 mg/kg of niacin, 4 times the 51 mg/kg found in the control diet and twice the value in beef diet (assays done by LARA Lab). Niacin can afford protection against carcinogenesis when added to a niacin-deficient diet (33), but high doses are toxic. Here, the high dose provided by the chicken-based diet would translate to 12 times the recommended daily allowance in humans. High niacin stimulates histamine release and prostanoid synthesis, which might explain the ACF promotion (34). The intake of white meat is not associated with colorectal cancer risk in most epidemiological studies (1,2). In contrast, dietary heme iron intake is associated with an increased risk of proximal colon cancer (35). However, in a prospective cohort study of 34,198 Californian Adventists, the consumption of white meat, mostly chicken, was associated with a tripled risk of colorectal cancer (36).

In summary, this study shows for the first time a promoting effect of red meat on carcinogenesis. It corroborates epidemiological observations: high red meat intake is associated with increased colon cancer risk. In previous meat studies (3–13), the promoting effect of meat was inhibited by dietary calcium, as shown by the study of Parnaud et al. (13). Furthermore, MDF promotion was related to heme intake. Promotion was significantly greater for the high-heme black pudding diet than for the medium-heme beef diet. This heme effect is in line with recent epidemiological data (35). The low-heme chicken diet did not promote MDF, but did increase ACF formation. For red meat diets, promotion was associated with high fecal water lipoperoxidation, cytolytic activity, and increase of pH, which may explain the increased carcinogenesis.

**ACKNOWLEDGMENTS**

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**LITERATURE CITED**

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