Influence of dietary protein on gastric tumorigenesis
by N-methyl-N'-nitro-N-nitrosoguanidine in rats

Part I Influence of various proportion of casein diets on
gastric tumorigenesis

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INTRODUCTION

A considerable amount of research has been reported on the significance of dietary
protein\(^1\)\(^2\) in the genesis and growth of certain neoplasms. Tannenbaum and
Silverstone\(^3\)\(^4\) first summarized the results obtained from the previous reports on the
relationship of dietary protein to the genesis and growth of tumors of spontaneous
origin and of those induced by chemical carcinogens. A more recent résumé by white\(^5\)
further reinforces the conclusion reached by Tannenbaum and Silverstone.

Although many efforts have been made to produce gastric neoplasms in laboratory
animals in the past; usually they have failed, i.e. experimental induction of gastric
adenocarcinoma of rodents has been especially difficult. Since a few instances of
induced adenocarcinoma of the glandular stomach in rats were reported by Stewart\(^6\)\(^7\),
Takayama et al\(^8\)\(^9\), many attempts have been made to obtain a high induction rate of
experimental carcinoma in the glandular stomach, but the results have not been
satisfactory.

Quite recently, Sugimura et al\(^10\)\(^11\)\(^12\), reported that a high induction rate of
adenocarcinoma of the glandular stomach in rats is producible by N-methyl-N'-nitro-N-
nitrosoguanidine (NG) administration.

It is thought that carcinogenesis of the gastro-intestinal tract, being the route of
entry of food, may be influenced by the proportion of dietary protein. However, so
far as we know, there have been no reports on the relationship between proportion of
dietary protein and carcinogenesis of the stomach in experimental animals.

The present experiment was designed to investigate the influence of the proportion
of casein diets on the incidence of gastric neoplasms induced by NG administration in
rats.

MATERIALS AND METHODS

Rats. Two hundred female Wistar rats (from Funabashi Farms, Chiba), usually 5 weeks old at the beginning of the experiment, were housed in groups of 5 to a wire cage in an airconditioned room kept at 24± 2 °C. They were given isocaloric experimental stock diets and drinking water supplemented with carcinogen ad libitum throughout their lives.

Experimental Diets. The compositions of the casein diets used in these experiments are shown in Table I. The experimental diets were isocalorically prepared in the form of tablets (about 2g.).

<table>
<thead>
<tr>
<th>Diet Composition</th>
<th>5% Casein Diet</th>
<th>20% Casein Diet</th>
<th>36% Casein Diet</th>
<th>50% Casein Diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein</td>
<td>5</td>
<td>20</td>
<td>36</td>
<td>50</td>
</tr>
<tr>
<td>Cornstarch</td>
<td>61</td>
<td>46</td>
<td>30</td>
<td>16</td>
</tr>
<tr>
<td>Sucrose</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>Cod liver Oil</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>Salts</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Vitamin mix.</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
</tbody>
</table>

Carcinogen. N-methyl-N'-nitro-N-nitrosoguanidine, obtained from Koch-Light Laboratories (Colonbrook, Bucks, England), was dissolved in drinking water in a concentration of 83 mg/liter.

Experimental Design. The experimental animals were divided into the following 4 groups:

Group I : Sixty rats were maintained on 5% casein diets and drinking water supplemented with NG.

Group II : Forty rats were maintained on 20% casein diets and drinking water supplemented with NG.

Group III : Sixty rats were fed on 36% casein diets and drinking water supplemented with NG.

Group IV : Forty rats were fed on 50% casein diets and drinking water supplemented with NG.

The animals were weighed at weekly intervals and kept till they died or were killed when obviously ill. On the 365th day all survivors were killed for necropsy. All that died or were killed before this date were also subjected to necropsy.
All the animals were carefully examined postmortem: not only the stomach but any other tissues showing abnormalities were fixed in 10% formalin and stained with Hematoxylin and Eosin, and Van Gieson's connective tissue stain. PAS stain and Alcian blue stain were used when required.

RESULTS

Changes in Body Weight. The body weight of the animals during the experiments are given in Fig. 1. The decrease in body weight of Group I (5% casein diets) was marked. Changes in body weight of rats of Group II (20% casein diets) was similar to those of Group IV (50% casein diets); i.e., weight gain was invariably noted in rats on diets of 20% casein and on 50% casein. However, rats on 36% casein diets gained weight for the initial four months, then gradually lost weight. The reason for this phenomenon is not known.

Gastric Lesions. The results are presented in Tables II to IV. Table II gives the incidence of gastric lesions and of neoplasms in all sites separately for each diet, and the effective number of animals per group. The occurrence of extragastric neoplasms will be discussed later. Table III shows the incidence of carcinoma of the glandular stomach along with the extent of neoplastic invasion of the wall of the stomach per group. Distribution of time required for development of carcinoma per group is shown in Table IV.
### Table II  Summary on the effect of proportion of casein diet on the genesis of gastric neoplasms induced by NG administration*

<table>
<thead>
<tr>
<th>Proportion of dietary casein</th>
<th>Initial No. of rats</th>
<th>Effective No. of rats</th>
<th>Carcinoma or atypical adenomatous neoplasm of glandular stomach</th>
<th>Polyp Leiomyoma total %</th>
<th>( p^{**} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>5%</td>
<td>60</td>
<td>0</td>
<td></td>
<td>0</td>
<td>N. S.</td>
</tr>
<tr>
<td>20%</td>
<td>40</td>
<td>21</td>
<td>6</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>36%</td>
<td>60</td>
<td>16</td>
<td>4</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>50%</td>
<td>40</td>
<td>20</td>
<td>14</td>
<td>0</td>
<td>1</td>
</tr>
</tbody>
</table>

* NG : N-methyl-N’-nitro-N-nitrosoguanidine (Koch-Light Laboratories, Colonbrook, Bucks, England)

** For the calculation of the statistical significance.

N. S. : not significant \((0.05<p)\).

### Table III  Summary on the effect of proportion of casein diet on the incidence of adenocarcinoma and/or atypical adenomatous neoplasm of glandular stomach induced by NG administration*

<table>
<thead>
<tr>
<th>Proportion of dietary casein</th>
<th>Initial No. of rats</th>
<th>Effective No. of rats</th>
<th>Extent of neoplastic invasion</th>
<th>total</th>
<th>%</th>
<th>( p^{**} )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>mucosal sub- intra-</td>
<td>muscular</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th>mucosal muscular</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>5%</td>
<td>60</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>20%</td>
<td>40</td>
<td>21</td>
<td>0</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>36%</td>
<td>60</td>
<td>16</td>
<td>0</td>
<td>0</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>50%</td>
<td>40</td>
<td>20</td>
<td>4</td>
<td>5</td>
<td>3</td>
<td>2</td>
</tr>
</tbody>
</table>

* NG : N-methyl-N’-nitro-N-nitrosoguanidine (Koch-Light Laboratories, Colonbrook, Bucks, England)

** For the calculation of the statistical significance.

N. S. : not significant \((0.05<p)\).

### Table IV  Average date of appearance for adenocarcinoma and atypical adenomatous neoplasm of glandular stomach in each group (month)

<table>
<thead>
<tr>
<th>Proportion of dietary casein</th>
<th>Average date of appearance for neoplasm</th>
<th>( p^{*} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>20%</td>
<td>10.66±1.88</td>
<td>N. S. [ p&lt;0.05 ]</td>
</tr>
<tr>
<td>36%</td>
<td>8.25±0.82</td>
<td>N. S. [ p&lt;0.05 ]</td>
</tr>
<tr>
<td>50%</td>
<td>9.42±2.06</td>
<td>N. S. [ p&lt;0.05 ]</td>
</tr>
</tbody>
</table>

* For the calculation of the statistical significance.

N. S. : not significant \((0.05<p)\).
**Group 1 (5% casein diets).** Among sixty rats, all animals died within six months. In none of the rats in this group were detected tumors of the stomach or other organs. In general, there was a marked atrophy of the glands in the mucosa of the glandular stomach of the animals which died within six months (Photo 1). In the mucosa of the forestomach no marked histopathological changes such as atypical hyperplasia could be observed.

**Group II (20% casein diets).** As may be seen in Table II, the effective number of rats which survived more than 7 months was 21. The first adenocarcinoma appeared in this group 12 months after initiation of experiment. Six of the effective 21 rats in this group had gastric neoplasms. Four of these tumors were adenocarcinoma and two were atypical adenomatous neoplasms without serous involvement. No metastases were observed.

As indicated in Photo 2, 2 rats sacrificed on the 365th day exhibited cauliflower-like tumors protruding into the lumen. Histologically, they were adenocarcinoma which penetrated the entire thickness of the gastric wall and extended to the serosal surface. Tumor areas showed positive staining with PAS (Photo 3). These features are in accord with the criteria of Stewart.

In 2 rats dying in the 12th month there were extremely atypical adenomatous growths infiltrating the muscularis propria. The muscle layer, where the tumors were situated, were atrophied due to the expansive growth. In the region of atypical adenomatous growth, atypical mitotic figures were numerous and atypism of tumor cells was detected frequently.

In 2 rats dying in the 8th month there was atypical hyperplasia of glands which invaded the muscularis mucosa.

**Group III (36% casein diets).** Forty-four rats were lost before the 7th month without tumor. Among the remaining 16 rats, there were six in which tumor was found. In two rats, adenomatous polyps were found. One of them dying in the 7th month had a polyp originating from the antral area (Photo 4). The other polyp was seen in the first portion of the duodenum of a rat dying in the 7th month.

The remaining 4 were histologically adenocarcinoma of the glandular stomach. The dates of appearance were as follows: one in the 7th month, one in the 8th month, two in the 9th month after initiation of the experiment. No metastases were detected. In this group, no tumor protruding into the gastric lumen was found.

In one rat of this group dying in the 7th month, atypical epithelial proliferation...
was seen in the submucosa invading the muscle layer. Mitotic figures and atypism of tumor cells were numerous.

In one rat dying in the 8th month, atypical adenomatous growth with many mitotic figures was found invading the submucosa and partly infiltrating the muscle layer.

In two rats dying in the 9th month, typical adenocarcinoma was found invading the muscularis propria of the glandular stomach.

In this group, no tumor was found in other organs.

**Group IV (50% casein diets)**. The highest incidence of neoplastic lesions in the glandular stomach was detected in this group. The effective number of rats was 20 and neoplastic lesions were found in the glandular stomach of 15. In one rat dying in the 7th month, a leiomyoma developed in the muscle layer of the glandular stomach (Photo 5). The remaining 14 rats had adenocarcinoma or atypical adenomatous neoplasm of the glandular stomach. The dates of appearance for tumors were as follows: three in the 7th month, four in the 8th month, one in the 9th month, one in the 10th month, five in the 12th month after initiation of experiment.

In 4 of 14 rats, neoplastic lesions protruded into the lumen of the glandular stomach when autopsied on the 365th experimental day (Photo 6). One of them had neoplastic invasion to the serosal surface, and chondrification in the interstitium of tumor tissue was seen. The chondrification showed positive staining with Alcian blue and PAS (Photo 7, and 8). In 2 of the remaining 3 rats, the invasion of the adenocarcinoma stopped at the muscle layer. The remaining rat sacrificed on the 365th day showed adenocarcinoma infiltrating throughout the muscle layer and reaching the serosal surface. In this case, atypical mitotic figures were numerous and atypism of tumor cells was detected frequently.

In one rat dying in the 10th month, atypical adenomatous growth with numerous mitotic figures infiltrated to the deep muscle layer.

The remaining rats had various grades of atypical glandular epithelial proliferation with atypism ranging from those limited to the glandular mucosa and submucosa to those partly infiltrating the muscle layer.

**DISCUSSION**

The role of dietary protein in the genesis of gastric cancer in man or experimental animals remains obscure.

Statistical analysis revealed a high incidence of gastric carcinoma among Japanese,
and low among Americans. In the past, many attempts to elucidate factors responsible for the high incidence of gastric cancer among Japanese have been directed toward determining whether Japanese food might potentiate carcinogenesis of the stomach. Yet, the mechanisms involved remain obscure. It is inferred that one reason may be that no valid experimental model for further investigation of gastric carcinogenesis has been provided. Recently, the high degree of carcinogenic potency shown by NG in the rat, and the apparent lack of carcinogenic activity on organs other than the glandular stomach and duodenum, makes this compound of great potential use as an experimental gastric carcinogen. But the mechanism involved in NG carcinogenesis is not clear. To clarify the possible factors in foodstuffs that accelerate carcinogenesis of stomach in rats on NG administration, Kagawa et al. did comparative studies on tumor incidence between diets with the composition of Japanese food (low calory, high salt) and of American food (high fat, low salt). A significantly high incidence of tumor in the gastrointestinal tract was noticed in the group subjected to Japanese diet compared with American diet. They suggested that the low incidence of neoplasms induced by NG on American diets is explained on the basis of a) their high fat content, b) the low salt content. With respect to the protein properties of both diets, there was slight difference between Japanese diets (12.3%) and American diets (14.6%). But the exact mechanisms involved in this result remain to be determined. To our knowledge, no detailed reports related to the influence of dietary protein on carcinogenesis of stomach in rats by NG have been described. The results obtained in this study show that none of the rats fed 5% casein diets developed neoplasms. The survival time of rats maintained on 5% casein diets was too short. No tumor developed among the rats that survived 5 to 6 months after beginning administration of NG. Our suggestion is that rats on 5% casein diets might also show induced gastric cancer, if the carcinogenic dosage were appropriately adjusted to allow longer survival of the rats. In contrast, among rats on moderate or high protein diets as many as 28.6 to 75% developed neoplasms, particularly carcinoma of the glandular stomach. These results suggest that high protein diets exert a potentiating effect on NG tumorgenesis. The difference between groups in incidence of histologically controlled tumors is given in Table II and III. There is no significant difference between group III (36% casein diet) and II (20% casein diet) in the ratio of carcinoma of glandular stomach to total number of effective rats, but there is some difference between these two groups if the number of benign tumors is considered. Group IV (50% casein diet), however, showed an acceleated

tumorgenesis. If the incidence of malignant tumor is considered, there is significant
difference between Groups IV and III or Groups IV and II.

If the average date of appearance of carcinoma is calculated, there is no significant
difference between Group III and IV, but there is significant difference between Groups
III and II (Table IV). NG has been known to be a very powerful mutagenic substance
since the report of Mandell and Greenberg\textsuperscript{14}. There are reports that the carcinogenic
action of NG\textsuperscript{14,15,16,17,18} may be due to modification of either protein or DNA.
However, the mechanism of the carcinogenic action of NG remains obscure.

From studies involving feedback inhibition, thymidine kinase possesses many of the
attributes of a rate-determining enzyme for DNA synthesis. It was reported that the
activity of thymidine kinase\textsuperscript{19} is enhanced in regenerating liver and in H35 hepatoma
on a high protein diet\textsuperscript{20}. Thus, presumably, the potentiating effects of high protein
diets may act not on the initiating stages of carcinogenesis but on the developmental
stages. Experiments are in progress that may reveal such a situation.

**SUMMARY**

The proportion of dietary protein modifies the tumorgenesis of glandular stomach in
rats that received NG in drinking water. If a 50\% casein diet is given regularly from
the date of carcinogenic stimulus by NG, the final tumor number and their malignancy
are more pronounced than in other groups maintained on 20\% or 36\% casein diets. 36\%
casein diets, when given concurrently with the carcinogen throughout the experimental
period, showed slightly more accelerated tumorgenesis than rats on 20\% casein diets,
but there were about the same number of malignancies. The survival time of rats on
5\% casein diets was too short and no tumors developed among rats that survived 5 to
6 months after experimental initiation.

The general conclusion drawn from these data is that high dietary protein exerts a
potentiation effect on NG tumorgenesis, especially in carcinogenesis.

**ACKNOWLEDGMENT**

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constant guidance and advice throughout this investigation, and to Prof. S. Morii,
Department of Pathology, for his valuable suggestions and advice on histopathological
examination.
References

19) Bollum, F. J., and Potter, V. R.: Nucleic Acid Metabolism in Regenearting Rat Liver VI.

Gastric Tumor of Rats feeding on 20% Casein Diets

Photo 1. H. E stain, ×40

Photo 2.

N-Methyl-N-nitroso-N’-nitroguanidine

Photo 3. PAS stain, ×12

Photo 7. Alcian blue stain, ×80

Photo 8. PAS stain, ×80