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The Effect of Interferon on Carcinogenesis by N-Ethyl-N′-nitro-N-nitrosoguanidine in the Duodenum of Mice

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The inhibitory effect of murine interferon α/β (Mu-IFN) on the induction of adenocarcinoma of the duodenum in C57BL/6 mice given N-ethyl-N′-nitro-N-nitrosoguanidine (ENNG) was examined. ENNG was given continuously in drinking water for 4 weeks and Mu-IFN was given intraperitoneally for 12 weeks. Then, the duodenal tumors of mice were examined stereomicroscopically and histologically. The level of IFN activity and natural killer (NK) activity were evaluated after an intraperitoneal single injection of Mu-IFN, and the level of NK activity was evaluated 2, 5 and 8 weeks after giving ENNG and Mu-IFN. In the mice given Mu-IFN, the incidence of duodenal tumors was significantly decreased ($P<0.01$), compared with that in mice given ENNG alone. Further, anti-asialo GM1 was given intraperitoneally every 5 days for 8 weeks to depress NK function and the incidence and size of duodenal tumors were examined. The results indicated that NK cells also have an important effect on the process of carcinogenesis. These data suggest that chemoprevention with IFN may be feasible.

Key words: Interferon — ENNG — Duodenal carcinogenesis — Natural killer cell

Interferon (IFN) is well known as an effective anti-cancer agent in mice and is considered to augment natural killer (NK) cell activity.[1-4] This study was designed in order to elucidate whether or not IFN has an inhibitory effect on the process of chemical carcinogenesis by N-ethyl-N′-nitro-N-nitrosoguanidine (ENNG) in the duodenum of mice. Several experiments have demonstrated that IFN can inhibit viral carcinogenesis and that IFN acts as an anti-viral agent.[5] If NK cells are activated by the administration of IFN to attack carcinogen-transformed cells, then IFN may be effective in inhibiting chemically induced carcinogenesis.

MATERIALS AND METHODS

Experimental animals Male C57BL/6 mice were purchased from Shizuoka Agricultural Cooperative Association for Laboratory Animals, Shizuoka. The mice were six weeks old and weighed about 20 g at the beginning of the experiment. They were fed with MF chow (purchased from Oriental Yeast Co., Ltd., Chiba).

Carcinogen ENNG (Nakarai Chemicals Ltd., Kyoto) was dissolved in tap water at 100 mg/liter or 500 mg/liter and the solution was given orally for four weeks as the drinking water. ENNG solution was freshly prepared three times a week and protected from light.

Experimental protocol A hundred and eighteen mice were divided into 5 groups, a control group (A), an IFN group (B), an ENNG group (C and C′), an ENNG with IFN group (D and D′), and an ENNG with anti-asialo GM1 group (E). The experimental protocol is shown in Fig. 1. All mice were killed in the 14th week and stereoscopic examination of the duodenal mucosa was performed. The incidence and size of tumors were examined under the stereomicroscope, and histological examinations were carried out.

Interferon Murine interferon α/β (Mu-IFN) was induced in a monolayer of L929 cells infected with Newcastle disease virus (Miyadera strain). The supernatant culture was kept for 72 h at 4°C to inactivate the residual virus. The supernatant solution was adjusted to pH 7, concentrated by ultrafiltration using cellophane tubing, dialyzed against pyrogen-free saline and stored at −80°C. The specific activity of the Mu-IFN was 0.5–1.0×10^7 IU/mg protein. The Mu-IFN (5×10^4 IU) was then dissolved in 1 ml of sterile saline prior to use and 5×10^4 IU/mouse of the Mu-IFN was administered intraperitoneally three times a week for twelve weeks in group B, D and D′ mice. At the same times, in group A, C and C′ mice, the same volume of saline was injected intraperitoneally as a control. The IFN assay was based on the reduction of the cytopathic effect on L929 cells infected with vesicular stomatitis virus.

Anti-asialo GM1 Asialo GM1 is a surface marker of the murine NK cells.[6] To suppress the function of NK cells, anti-asialo GM1 (Wako Pure Chemical Industries, Ltd., Osaka) was dissolved in saline and 30 μg/0.3 ml of

Abbreviations used are: Mu-IFN, murine interferon; ENNG, N-ethyl-N′-nitro-N-nitrosoguanidine; NK, natural killer; anti-AGM1, anti-asialo GM1.

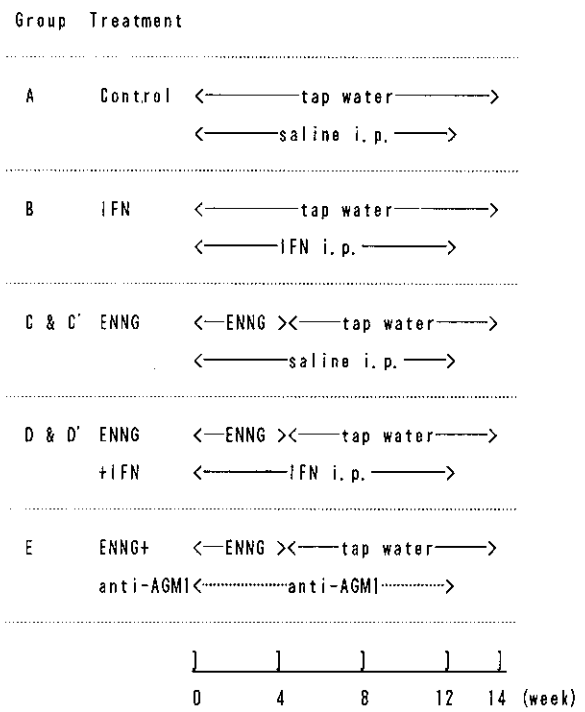

Fig. 1. Experimental schedule. IFN: Mu-IFNα/β, 5×10^4 IU, 3 times/week i.p. Saline: 3 times/week i.p. ENNG: 100 mg/liter (groups C and D) or 500 mg/liter (groups C′ and D′) p.o. Anti-asialo GM1: 30 μg, every 5 days i.p.

anti-asialo GM1 was administered intraperitoneally every five days for eight weeks to the mice in group E.

Natural cytotoxicity assay According to the method described by Tracy *et al.*,[7] YAC-1 cells (T cell lymphoma induced by Moloney leukemia virus) were maintained in suspension cultures in RPMI-1640 medium supplemented with 10% fetal bovine serum. Then, 2×10^6 YAC-1 cells were suspended in 0.1 ml of RPMI-1640 medium, containing 50 μCi of $Na_2{}^{51}CrO_4$ and incubated for one hour at 37°C. The labeled cells were washed three times, diluted in RPMI-1640 medium containing 10% fetal bovine serum and 10 m*M* HEPES buffer and adjusted to 10^5 cells/ml for use as the target cell suspension. Effector cells were obtained from mouse spleen in each of the experimental groups. The spleen was minced with scissors, and spleen cells were suspended in the medium and incubated on the plastic dish at 37°C for 1 h in a 5% CO_2 atmosphere to remove the spleen macrophages. These effector cells were washed and adjusted to 10^7 cells/ml in RPMI-1640 medium, containing 10% fetal bovine serum and 10 m*M* HEPES buffer. Then, 100 μl of labeled target cell suspension and the same volume of effector cell suspension were added to the wells of round-bottomed Micro Test II dishes. The cells were set up in triplicate and incubated at 37°C for 4 h in a 5% CO_2 atmosphere. Then, aliquots of 100 μl of the supernatants were removed and counted in a Beckman gamma counter. Spontaneous release (background), computed from wells containing target cells with only complete medium, was always less than 10% of the total count obtained from wells in which the target cells were lysed with 1% Triton 100X (Nakarai Chemicals Ltd.) in RPMI-1640 medium. Percent release was calculated according to the following formula:

$$\text{NK activity (\%)} = \frac{\text{test cpm} - \text{spontaneous cpm}}{\text{total cpm} - \text{spontaneous cpm}} \times 100.$$

These measurements of NK activities were performed in the 2nd, 5th and 8th weeks of this experiment.

Stereoscopic and microscopic findings of the duodenal tumors All mice were killed in the 14th week, and the duodenum were resected and fixed with 10% formalin, then stereoscopic examination of the duodenal mucosa was performed. The tumors were observed as polyp-like enlarged villi or crater-form tumors. The incidence and size of tumors were counted and measured under the stereomicroscope. Then, the tumors were fixed immediately, dehydrated and embedded by routine procedures. Most sections were stained with hematoxylin-eosin for histological examination.

Statistical analysis Student's *t* test was used for statistical analysis.

RESULTS

Effects of Mu-IFN on NK cell function The effects of Mu-IFN on changes in the activity of NK cells were measured. A single dose of 5×10^4 IU of Mu-IFN was administered intraperitoneally, and the NK activity and serum Mu-IFN levels were measured simultaneously at suitable intervals. The results are shown in Fig. 2. The Mu-IFN level in the serum increased rapidly after the intraperitoneal injection of Mu-IFN, reaching a peak after one hour, then rapidly decreased. However, a low level of Mu-IFN was still detected 24 h later. The level of NK activity was low before the Mu-IFN administration, but gradually increased to reach a peak at 6 h. A high level was maintained for more than 24 h.

NK activity The NK activities in each group were measured in the 2nd, 5th and 8th weeks of this experiment. It is considered that NK activity is age-dependent, so mice without any treatment were used as controls. The results are shown in Fig. 3. In the control group, the NK activities always remained low. The Mu-IFN-treated mice showed higher levels than the other experimental groups except for the mice which were administered both ENNG and Mu-IFN. The ENNG-treated mice showed high levels of NK activity, like the Mu-IFN-treated mice,

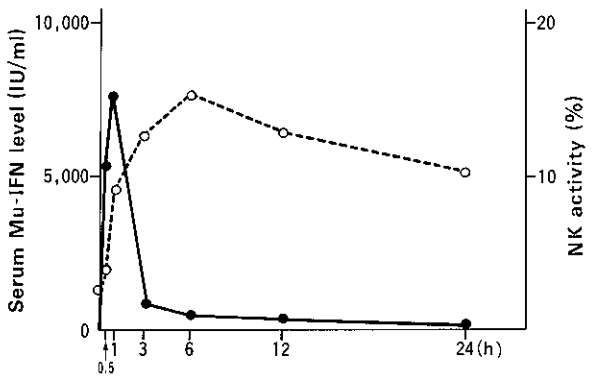

Fig. 2. Change of serum Mu-IFN levels and NK activity of spleen cells after single intraperitoneal injection of Mu-IFN α/β (5×10^4 IU/mouse). ●, Serum Mu-IFN level; ○, NK activity.

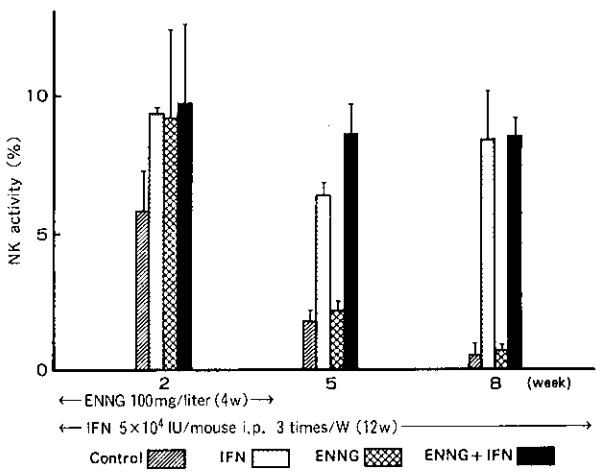

Fig. 3. Change of NK activity of spleen cells during the administration of ENNG and Mu-IFN α/β.

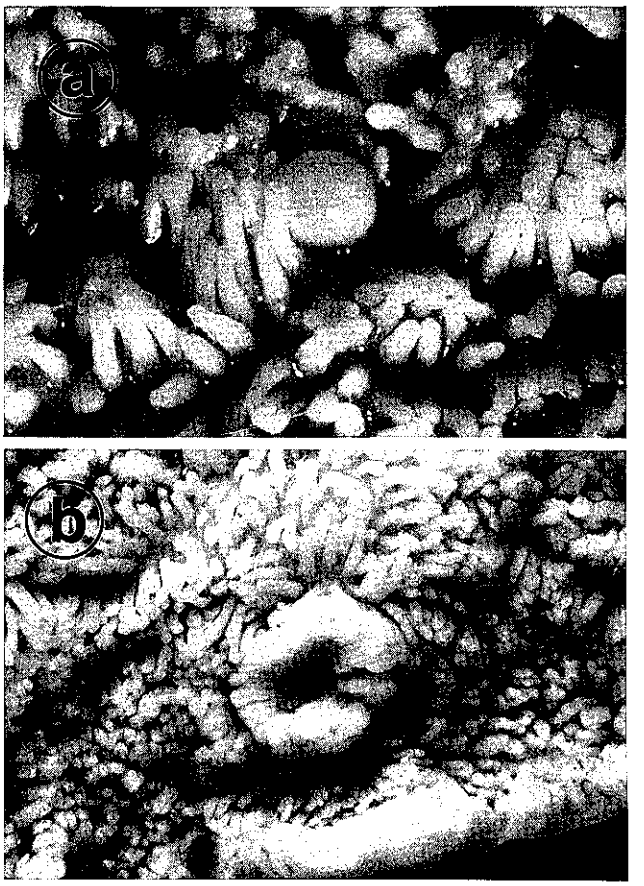

Fig. 4. Stereoscopic findings of the duodenal tumors (×40). a: Small tumor similar to an enlarged duodenal villous among normal villi. b: Large tumor with a depression at the center.

in the 2nd week, but the activity decreased markedly thereafter. Similar declines of NK activity with time were seen in both ENNG-treated mice and control mice. However, the ENNG with Mu-IFN group maintained higher levels than the other groups and the NK activity of this group was not suppressed by the administration of the carcinogen.

Stereoscopic and microscopic findings of the duodenal tumors All mice were killed on the 14th week, and stereoscopic examination of the duodenal mucosa was performed. Small tumors resembling enlarged duodenal villi were observed among the other normal villi, and large tumors were observed with a depression at the center (Fig. 4). Histologically, the tumors in the duodenum consisted of atypical glands and basophilically stained cells with a large number of mitotic bodies. The nuclei were large, and were located at different levels in the cytoplasm. These tumor cells were consistent with adenocarcinoma. Small foci of adenocarcinoma are shown in Fig. 5a, and a large tumor, which has invaded the submucosal space, in Fig. 5b.

Incidence and size of duodenal tumors The tumors on a 4 cm length of duodenum from the pyloric ring were counted and measured under a stereomicroscope. Table I shows the incidence and size of duodenal tumors when 500 mg/liter of ENNG was administered. There was no statistically significant difference in the incidence of tumors between the ENNG group (C′) and the ENNG with Mu-IFN (D′) group. Table II shows the incidence and size of duodenal tumors when 100 mg/liter of ENNG was administered. In group C of the ENNG-treated mice, seventeen of twenty mice bore duodenal tumors. However, in group D of ENNG with Mu-IFN

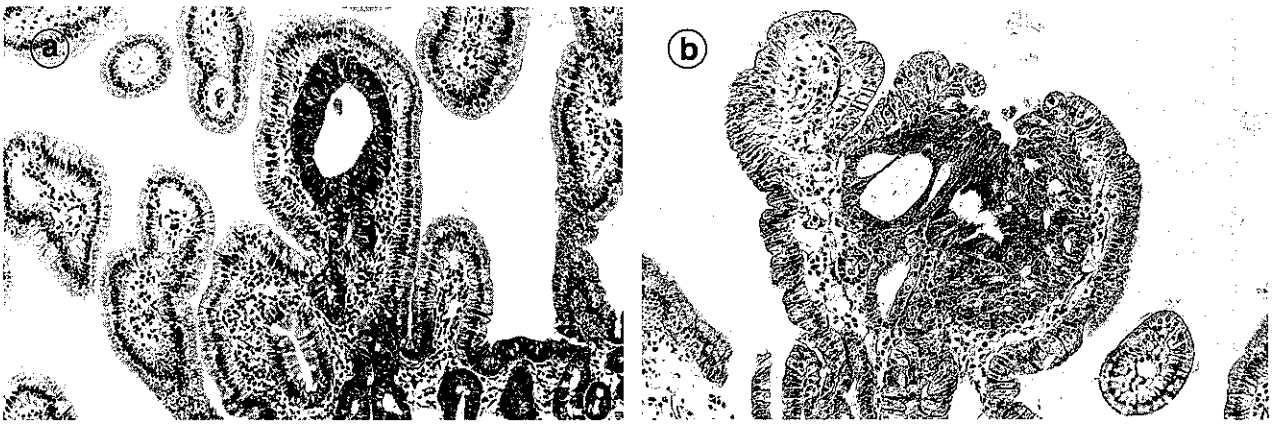

Fig. 5. Histological findings of the duodenal tumors (hematoxylin-eosin staining, ×100). a: Small foci of adenocarcinoma in the submucosal layer. b: Large tumor, which has invaded the submucosal space.

Table I. Incidence and Size of Duodenal Tumors in Mice Given ENNG (500 mg/liter) for 4 Weeks and Mu-IFN α/β

Group	A Control	B IFN	C′ ENNG	D′ ENNG+IFN
Number of mice	9	9	24	24
Mice with tumors	0	0	23[a)]	20[a)]
Tumor incidence (%)	0	0	96	83
Total tumors	0	0	78	68
Tumor per mouse (mean±SD)	0	0	3.3±2.1	2.8±2.5
Diameter of tumor (mm, mean±SE)	0	0	0.96±0.07	1.21±0.11

a) No significant difference.

Table II. Incidence and Size of Duodenal Tumors in Mice Given ENNG (100 mg/liter) for 4 Weeks and Mu-IFN α/β

Group	A Control	B IFN	C ENNG	D ENNG+IFN	E ENNG+IFN +anti-AGM1
Number of mice	9	9	20	20	12
Mice with tumors	0	0	17	9	12
Tumor incidence (%)	0	0	85[a, c)]	45[a)]	100[c)]
Total tumors	0	0	37[b)]	15[b)]	30
Tumors per mouse (mean±SD)	0	0	1.9±1.1	0.8±1.1	2.5±1.2
Diameter of tumor (mm, mean±SE)	0	0	0.67±0.11	0.80±0.17	0.56±0.05

a), *b*) $0.005 < P < 0.01$.
c) Not significantly different.

mice, nine of twenty mice had duodenal tumors, and eleven of the mice were free from tumors. The difference between group C and group D is statistically significant. However, the size of tumors was not decreased significantly.

Anti-asialo GM1 treatment In the anti-asialo GM1 group administered with ENNG (group E), the incidence of duodenal tumor was 100%, which is higher than in the group treated with ENNG alone (group C).

DISCUSSION

It is well known that IFN has some anti-tumor effect. Some reports have described IFN as having an inhibitory effect on the process of carcinogenesis,[8,9] but these reports dealt with viral carcinogenesis and the effect of IFN may be due to direct inhibition of viral growth. However, IFN also activates NK cells and macrophages, enhancing cellular immunity. In this study, we investigated the role of NK activity in the anti-promotive effect of IFN, which activates NK cells without stimulation by tumor-associated antigen,[10,11] in a chemical carcinogenesis model using ENNG in mice.

We used C57Bl/6 mice for these experiments because a suitable experimental model has been developed by Matsuyama *et al.*[12] In this model, ENNG was used as a chemical carcinogen and it was administered per orally. This model is convenient to study the early stages of carcinogenesis. The tumors induced in this experiment were classified into three types. The first is the enlargement of a single villus, the second is the fusion of several villi and the third is a crater-form lesion. The intravillous lesions develop into macroscopic crater-form lesions, so it is easy to compare the incidence of carcinogenesis in the early stages by counting the number and size of these intravillous lesions.

Carcinogens usually suppress the host immunity.[13,14] In the present study, ENNG also suppressed the NK activity of the host. But, in addition, the NK activity declined with time. On the other hand, it has been reported that the administration of IFN activates the NK activity of the host.[4] In this study, the administration of IFN resulted in high levels of NK activity, even when ENNG was administered simultaneously.

In the 500 mg/liter ENNG group, the tumor incidence was decreased by the administration of the IFN, but without statistical significance. In the 100 mg/liter group, the tumor incidence was significantly suppressed by the administration of the IFN. The NK activity was maintained at a high level in both groups.

When anti-asialo GM1 was used to depress the function of NK cells,[15] the incidence of duodenal tumor increased, implying that NK cells played a role in the defense against duodenal carcinogenesis.

In conclusion, immune responsiveness of the host and non-specifically immune-competent NK cells do play a role in the response to chemical carcinogenesis with ENNG in mice IFN activates not only the function of the NK cells but also the function of macrophages,[16] and NK activity was enhanced by IFN at both concentrations of ENNG tested. However, tumor incidence was not reduced significantly at the higher dose of 500 mg/liter of ENNG.

It is possible that in tumor-free cases, the target cells or host were sensitive to IFN, whereas in tumor-producing mice, they were insensitive or less sensitive. Since inhibition of carcinogenesis by IFN is not so potent, the IFN administration may not be effective in inhibiting carcinogenesis, if a high dose of chemical carcinogen is applied, and other biological response modifiers may have the same drawback. A high dose of IFN in conjunction with a low dose of carcinogen is necessary to detect the inhibition of carcinogenesis by IFN. These data suggest that chemoprevention with IFN may be feasible.

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