Suppression of Antibody-mediated and Cell-mediated Murine Immunity by the Carcinogen N-[4-(5-Nitro-2-furyl)-2-thiazolyl]acetamide

Don B. Headley, Samuel M. Cohen,2 and George T. Bryan3

Department of Human Oncology, University of Wisconsin Center for Health Sciences, Madison, Wisconsin 53706

SUMMARY

N-[4-(5-Nitro-2-furyl)-2-thiazolyl]acetamide (NFTA) administered at 100 ppm in diet to mice for 12 weeks induced a high incidence of lymphocytic leukemia. Effects of NFTA on antibody-mediated immunity and cell-mediated immunity of BALB/c mice were studied using the spleen plaque assay for detection of immunoglobulin M-producing cells and the graft-versus-host (GVH) reaction, respectively. NFTA suppressed both responses. With the spleen plaque assay, the number of antibody-forming cells (AFC) to sheep red blood cells was significantly less than in unmedicated, control mice after treated mice received NFTA at 1000 ppm for 6 days. The GVH reaction was not suppressed at 70 days, prior to the histological appearance of leukemia. Effect of dose was studied by administering NFTA at 100, 250, 500, and 1000 ppm of diet for 13 to 14 weeks and then determining the response in the spleen plaque assay and GVH reactions. The ratio of AFC/spleen was 34% ± 11% for those with leukemia compared with 170 x 10^3 ± 74 for those without leukemia, p < 0.01 for both groups, compared with controls. A closely related carcinogenic nitrofuran, N-[4-(5-nitro-2-furyl)-2-thiazolyl]formamide, did not suppress the antibody-mediated immunity response measured during the 11th week of administration.

INTRODUCTION

NFTA, an effective antibacterial drug used to treat human infectious diseases in some countries (11, 25), has demonstrated carcinogenic activity in mice (7, 10), rats (15), hamsters (12), and dogs (14) with differing organ specificity in different species. In several strains of mice, it induced high incidences of thymic lymphoma with lymphocytic leukemia and low incidences of forestomach squamous cell carcinomas. The leukemia was characterized by large thymus, spleen, and lymph nodes; by lymphocytic infiltration of most other tissues; and by bone marrow replacement by leukemic cells with a greatly increased number of lymphocytes in the peripheral blood. In BALB/c mice, the leukemia was induced in 12 to 16 weeks, was completely inhibited by thymectomy at 3 to 4 weeks of age, but was unaffected by splenectomy at 3 to 4 weeks of age (8). The forestomach tumors, though, were more numerous and invasive when thymectomy, but not splenectomy, was performed.

NFTA is one of numerous 5-nitrofurans that have demonstrated carcinogenic activity in experimental animals (6, 30) and mutagenic activity in microorganisms (29, 36, 37). Other classes of chemical carcinogens, such as the polycyclic hydrocarbons (26), aromatic amines (27), and nitroquinoline-N-oxides (21), have also demonstrated mutagenic activity and frequently have been immunosuppressive when tested in mice (2, 32). NFTA was tested (9, 19) for its effects on the immune system as a representative of the nitrofuran class of carcinogens because of its demonstrated effect on the lymphocytic cell population in mice. A structurally related nitrofuran, FANFT, induced urinary bladder carcinomas in rats, mice, hamsters, and dogs (12, 14, 16–18), and its effect on the AMI of mice was also tested.

MATERIALS AND METHODS

Mice. Inbred female BALB/c mice were obtained from The Jackson Laboratory, Bar Harbor, Maine, at 5 weeks of age and served as the experimental animals for the studies performed. BALB/c x A/J F1 (hereafter called CAF1) mice were also obtained from The Jackson Laboratory and were

1 Supported in part by Grants CA 10017, CA 11946, and CA 14520 from the National Cancer Institute, USPHS. Preliminary reports of this work have been made (9, 19).
2 Present address: 5 Woodford Street, Worcester, Mass. 01604.
3 To whom requests for reprints should be addressed, at Department of Human Oncology, University of Wisconsin Center for Health Sciences, 1300 University Avenue, Madison, Wis. 53706.
Received August 2, 1976; accepted December 20, 1976.
used as host mice and negative control donor mice for the GVH assay.

**NFTA Administration to Mice.** NFTA was received as a gift from U. Ravizza, Milan, Italy, and its identity and purity were determined by melting point, by IR and UV spectrophotometry, and by paper chromatography in a solvent system of methanol:1:butanol:benzene:water (2:1:1:1) and the same solvent system plus 1% glacial acetic acid (3, 28). It was mixed in the diet (ground Wayne Lab Blox, Allied Mills, Inc., Chicago, Ill.) at doses of 100, 250, 500, and 1000 ppm (w/w), as described (30), and was administered p.o. for varying periods of time to appropriate groups of mice upon arrival. The day feeding was begun was considered Time 0 of the experiments. Untreated control groups received only the ground diet with no added chemical.

**Measurement of AFC by Spleen Plaque Assay.** Sheep blood in Alsever’s solution (Grand Island Biological Co., Grand Island, N.Y.) was washed 3 times in 0.9% NaCl solution, and the packed cells were resuspended in 0.9% NaCl solution to give a 10% suspension of SRBC. The mice were immunized by injection of 0.5 ml of 10% SRBC i.p. 5 days prior to removal of the spleen for the spleen plaque assay. The spleen plaque assay used was a further modification of Cunningham and Szenberg’s (13) slide method of Jerne et al. (22), using 2 glass slides taped together rather than a slide and coverslip. IgM-producing AFC were counted after 1 hr of incubation at 37° and the number of nucleated spleen cells were counted in a hemocytometer following crystal violet staining.

**Estimation of CMI by GVH.** The GVH reaction was determined by the method of Simonsen et al. (33, 34). The donor mice for the positive control group (strong GVH reaction) were the untreated BALB/c mice fed control diet, and they were of the same age as the NFTA-fed donor mice. CAF, female mice of the same age as the NFTA-fed donor mice served as the negative control donor group (weak GVH reaction), and 2- to 3-day-old CAF, mice served as host mice. For each assay, 2 positive control, 3 experimental, and 1 negative control donor mice were used, and 107 spleen cells from each donor mouse were injected i.p. into each of the 4 to 6 host mice. Ten days after injection, the recipients were weighed, their spleens and livers were removed and weighed, and partial body weight was determined. Spleen indices were calculated by the method of Simonsen et al. (33, 34).

**Statistics.** The unpaired Student’s t test was used for statistical analysis of the data from the GVH and spleen plaque assay experiments (35). Spleen indices greater than 1.30 were statistically significant (p < 0.05) compared with negative control groups, utilizing animal populations of 10 to 15/group. To ascertain whether a dose-response relationship existed with immunosuppression as assessed by the GVH, the slope, b, of the regression equation best representing the linear relationship was computed by the method of least squares and was tested for statistical significance by the F test (35).

**Comparison of NFTA Immunosuppression and Leukemogenicity.** Sixty BALB/c female mice, 5 weeks of age, were divided into 2 groups of 30 mice each. One group received NFTA at a dose of 500 ppm of the diet for 14 weeks, followed by unmedicated control diet for 14 weeks, and the other group received only control diet. During the 11th week, SRBC were injected i.p. into all mice, and 5 days later the spleens were surgically removed and the spleen plaque assay was performed. As mice died or were killed after 28 weeks, when the experiment ended, an autopsy was performed and tissues were processed as previously described (30). All histological sections were stained with hematoxylin and eosin.

**Effect of FANFT on AMI.** FANFT was obtained from Saber Laboratories, Morton Grove, Ill., and was administered in the diet at a dose of 940 ppm (equimolar to 1000 ppm NFTA) to 40 female 5-week-old BALB/c mice for 30 weeks, followed by control diet for 22 additional weeks, at which time the surviving mice were killed and examined for tumors. During the 11th week of FANFT administration, 10 mice were tested by spleen plaque assay for effects on AMI. A comparable group of 40 mice served as controls, and 10 mice from this group were tested simultaneously by spleen plaque assay.

**RESULTS**

**Relationship between Time of NFTA Administration and Suppression of AFC.** In order to determine the time course and extent of suppression of AFC, NFTA was fed at 1000 ppm of the diet to BALB/c female mice at 5 weeks of age for a maximum of 14 weeks. Five NFTA-fed and 5 unmedicated control-fed mice were immunized with SRBC 5 days prior to killing and analysis by the spleen plaque assay (Table 1). There was a progressive decline in the AFC/spleen in mice administered NFTA and, by the 6th day after initiation of NFTA feeding, the difference compared with the controls was statistically significant (p < 0.05). Suppression of AFC/spleen was significantly less than controls at each time period tested beyond 5 days of NFTA administration. The value at 208 days represents a time period 15 weeks beyond cessation of NFTA administration. Although NFTA suppressed the number of spleen cells, it appeared to selectively suppress AFC to a greater degree as the number of AFC/106 nucleated spleen cells gradually decreased, becoming statistically significant by the 7th day of NFTA exposure.

**Relationship between Time of NFTA Administration and Suppression of GVH Reaction.** To determine the course and extent of suppression of the GVH reaction, NFTA was fed to BALB/c mice at a dose of 1000 ppm through the time of killing at Days 1, 7, 21, and 70 (Chart 1). There was no significant suppression of the GVH reaction by 21 days, but it was severely suppressed by Day 70 (p << 0.001).

**Relationship between Dose of NFTA and Suppression of AFC.** NFTA was administered to groups of 5 female 5-week-old mice at doses of 100, 250, 500, and 1000 ppm of the diet through the time of killing. During Weeks 13 and 14, the mice were immunized with SRBC and the spleen plaque assay was performed 5 days later. The spleen plaque assay was performed on groups of control mice simultaneously with test mice (Table 2). Significant suppression of the number of AFC per spleen was present with doses of 250,
Table 1

Temporal effect of NFTA administration on the humoral immune response
Mice were fed either control diet or control diet with 1000 ppm NFTA ad libitum. Initiation of NFTA-medicated diets began on day 0 when mice were 5 weeks old. Groups of 5 mice were given i.p. injections of SRBC at different time intervals throughout the feeding and assayed by spleen plaque assay 5 days later. NFTA was fed until mice were killed, except where noted.

<table>
<thead>
<tr>
<th>Days mice received NFTA*</th>
<th>AFC/spleen</th>
<th>AFC/10⁶ spleen cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>NFTA</td>
</tr>
<tr>
<td>1</td>
<td>54.6 ± 13.8</td>
<td>43.7 ± 6.0</td>
</tr>
<tr>
<td>2</td>
<td>42.9 ± 7.7</td>
<td>31.6 ± 11.3</td>
</tr>
<tr>
<td>4</td>
<td>53.8 ± 21.4</td>
<td>38.5 ± 9.4</td>
</tr>
<tr>
<td>5</td>
<td>50.7 ± 27.1</td>
<td>24.5 ± 21.1</td>
</tr>
<tr>
<td>6</td>
<td>72.4 ± 38.3</td>
<td>29.3 ± 14.1</td>
</tr>
<tr>
<td>7</td>
<td>57.2 ± 22.5</td>
<td>16.5 ± 10.9</td>
</tr>
<tr>
<td>12</td>
<td>82.3 ± 45.0</td>
<td>10.7 ± 7.4</td>
</tr>
<tr>
<td>19</td>
<td>99.8 ± 65.2</td>
<td>15.3 ± 12.7</td>
</tr>
<tr>
<td>75</td>
<td>146.6 ± 36.4</td>
<td>79.0 ± 39.8</td>
</tr>
<tr>
<td>208d</td>
<td>123.5 ± 50.9</td>
<td>28.0 ± 36.0</td>
</tr>
</tbody>
</table>

* Days mice received NFTA prior to killing and performance of the spleen plaque assay. The mice were killed 5 days after immunization.

Table 2

Dosage effect of NFTA on the AMI
Groups of 5 mice were fed NFTA at the doses indicated, or unmedicated control diet, and were given i.p. injections of SRBC between Weeks 13 and 14 and killed for spleen plaque assay 5 days later.

<table>
<thead>
<tr>
<th>Dose (ppm)</th>
<th>Total NFTA consumed (mg)</th>
<th>(AFC/spleen) NFTA</th>
<th>(AFC/spleen) control</th>
<th>p*</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>26</td>
<td>0.86 ± 0.21</td>
<td>&lt;0.30</td>
<td></td>
</tr>
<tr>
<td>250</td>
<td>100</td>
<td>0.22 ± 0.06</td>
<td>&lt;0.01</td>
<td></td>
</tr>
<tr>
<td>500</td>
<td>208</td>
<td>0.33 ± 0.13</td>
<td>&lt;0.01</td>
<td></td>
</tr>
<tr>
<td>1000</td>
<td>304</td>
<td>0.54 ± 0.12</td>
<td>&lt;0.01</td>
<td></td>
</tr>
</tbody>
</table>

* Values of p listed are for the given doses versus the control group. The p values for comparisons between doses (ppm) are: 100 versus 250, p < 0.01; 100 versus 500, p < 0.01; 100 versus 1000, p < 0.02; 250 versus 500, p < 0.02; 250 versus 1000, p < 0.01; 500 versus 1000, p < 0.05.

500, and 1000 ppm but not at 100 ppm. The degree of suppression was greatest at a dose of 250 ppm and became less with higher doses. The differences at the 3 higher doses were significant between groups (see Table 2).

Relationship between Doses of NFTA and Suppression of the GVH Reaction. Groups of female 5-week-old BALB/c mice were fed NFTA for 10 weeks at the same doses as for the spleen plaque assay. The GVH reaction results are shown in Chart 2. Significant suppression of the GVH reaction, compared with the positive control animals at each dosage level, was evident at the 2 highest doses, 500 (p < 0.05) and 1000 (p < 0.001) ppm. The degree of suppression was directly related to dose, as demonstrated by the linear regression analysis. Slope, b, of the linear regression line for NFTA-fed mice was statistically significant (p < 0.05).

Comparison of NFTA Immunosuppression and Leukemogenicity. At 500 ppm NFTA, 8 of 18 mice developed thymic lymphomas with leukemia, and 2 of 18 developed small stomach papillomas, as described previously (8). The responses of the 8 mice with leukemia and the 10 NFTA-fed mice without leukemia to SRBC, as measured by the spleen plaque assay, were compared and each was compared with the control group of 27 mice (Table 3). Both subgroups fed NFTA were significantly different with respect to AFC/spleen, compared with the control group (p < 0.01 in each instance), but were not different when compared with each other (0.6 > p > 0.5).

FANFT Carcinogenicity and Effect on AMI. Twenty-two of 24 BALB/c mice fed FANFT for 30 weeks developed carcinomas of the urinary bladder during the subsequent 22 weeks on control diet, the histology being similar to that

---

D. B. Headley et al.

---

Chart 1. The effect of NFTA administration on the CMI response of BALB/c mice as determined by the GVH reaction. For each assay, 2 positive control, 3 experimental, and 1 negative control donor mice were used, and 10⁶ cells were injected i.p. into each of 4 to 6 host mice for each donor mouse.

---

CANCER RESEARCH VOL. 37
The relationship of NFTA-induced leukemia and AMI immunosuppression as measured by the spleen plaque assay

Mice were fed either unmedicated control diet or NFTA at 500 ppm for 14 weeks. After 11 weeks on the respective diets the mice were injected with SRBC and 5 days later were splenectomized for the spleen plaque assay. The spleen plaque assay results for individual mice that received NFTA were combined into either with-leukemia or without leukemia categories when the mice were killed 28 weeks after feeding began and tissues were microscopically examined.

Table 3

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of mice</th>
<th>No. of mice with stomach papillomas</th>
<th>AFC/spleen</th>
<th>p&lt;sup&gt;a&lt;/sup&gt;</th>
<th>AFC/10&lt;sup&gt;6&lt;/sup&gt; spleen cells</th>
<th>p&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>27</td>
<td>0</td>
<td>170 ± 74&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
<td>1310 ± 600</td>
<td></td>
</tr>
<tr>
<td>NFTA at 500 ppm, without leukemia</td>
<td>10</td>
<td>1</td>
<td>68 ± 24</td>
<td>0.01&lt;sup&gt;d&lt;/sup&gt;</td>
<td>750 ± 240</td>
<td>&lt;0.01&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>NFTA at 500 ppm, with leukemia</td>
<td>8</td>
<td>1</td>
<td>78 ± 34</td>
<td>0.01&lt;sup&gt;d&lt;/sup&gt;</td>
<td>680 ± 480</td>
<td>0.10 &gt; p</td>
</tr>
</tbody>
</table>

<sup>a</sup> AFC x 10<sup>3</sup>/spleen.
<sup>b</sup> p value compares statistical significance of untreated control versus treated groups.
<sup>c</sup> Mean ± S.D.
<sup>d</sup> NFTA mice with leukemia versus those without leukemia are statistically similar (0.6 > p > 0.5).
<sup>e</sup> NFTA-fed mice with leukemia versus those without leukemia are statistically similar (0.5 > p > 0.4).

DISCUSSION

Numerous 5-nitrofurans have demonstrated carcinogenic activity in experimental animals (6) and mutagenic activity in microbial test systems (29, 36, 37). The effects of NFTA on the lymphoid system in mice suggested that immunosuppression may play a role in the development of the resulting leukemia. Adult thymectomy before NFTA administration prevented leukemia, demonstrating that it was thymus dependent; but adult splenectomy had no apparent effect on leukemogenesis (8). The immunosuppressive effects of NFTA at leukemogenic doses and at a time interval before the appearance of leukemia for both the AMI and CMI have been demonstrated in this report.

The early and severe suppression of AMI in NFTA-fed mice suggests that the AMI suppression greatly antedates any visible leukemic change caused by NFTA, and, therefore, does not seem to be a side effect of the resulting leukemia. However, no difference in the degree of AMI suppression at 11 weeks was noted in NFTA-fed mice that developed leukemia, compared with leukemia-free NFTA-fed mice. Thus, although NFTA suppressed the AMI, the suppression did not appear to correlate with the development of leukemia.

The CMI is considered of greater importance in relation to tumorigenesis (20), and several chemical carcinogens (2) and oncogenic viruses (5) have demonstrated suppressive activity of the CMI. NFTA suppression of CMI was directly related to the dose and correlates with the dose dependency of leukemia in NFTA-fed mice (7). Depression of the CMI was not observed until several weeks prior to the first visible signs of leukemia. It is possible that the leukemia was already developing by the time the 1st CMI suppression was noted. It is also quite possible that the CMI could have been suppressed quite early in the feeding, like the AMI, but the GVH was not sufficiently sensitive to reveal these less severe suppressions of the CMI. The hypothesis that immunosuppression precedes the leukemogenic changes brought about by NFTA seems most consistent with the data.

Considerable experimental and clinical evidence suggests that suppression of the immune response results in greater incidences of cancer with shorter latent periods. Patients with congenital immunodeficiencies (24) and organ transplant recipients receiving immunosuppressive therapy (31) have increased incidences of tumors, particu-
larly malignant lymphomas. Numerous chemical carcinogens (2) and oncogenic viruses (5) have demonstrated immunosuppressive activity, usually well before development of tumors. Other carcinogenic nitroaromatic compounds, such as 4-nitroquinoline N-oxide, may suppress only the humoral and not the cellular immune response (32), further complicating the postulated relationship between immunosuppression and carcinogenesis. Also, the relationship between generalized immunosuppression, as measured by the assay systems we have used, and specific suppression of immune response to tumor antigens is not known. We have been unable thus far to show evidence of tumor-specific transplantation antigens on NFTA-induced lymphocytic leukemia. Alternative hypotheses to the mechanism of chemical carcinogenesis, such as activation of a leukemogenic virus or altering the susceptibility of these lymphoid cells to viral infection, have been proposed (4, 7, 23).

NFTA demonstrated immunosuppressive activity and is but one of many 5-nitrofurans which demonstrated carcinogenic and mutagenic activity. The carcinogen FANFT, which is also a mutagen (29, 37), appears to be quite specific for inducing carcinomas of the urinary bladder with little or no leukemogenic activity in mice (10, 16). Unlike NFTA, FANFT did not suppress the humoral immune response in BALB/c mice when measured at only 1 time interval, 11 weeks. Similarly, FANFT fed to rats induced bladder carcinomas (17, 18), and no suppressive activity was demonstrated when the AMI (as measured by the spleen plaque assay) and CMI (as measured by the phytohemagglutinin stimulation) were tested at 3 time intervals (1).

Immunosuppression by NFTA may be related to its leukemogenic activity, and it remains to be seen whether other carcinogenic 5-nitrofurans inducing other types of tumors have such activity.

ACKNOWLEDGMENTS

We are grateful to Dr. Robert Auerbach (Department of Zoology, University of Wisconsin) for his advice and suggestions. We thank A. Hussbaum, J. Davis, K. Bloomer, and N. Worley for diet preparation, care of mice, and assistance with assay procedures, and K. Deighton and M. Post for assisting in the preparation of the manuscript.

REFERENCES

Suppression of Antibody-mediated and Cell-mediated Murine Immunity by the Carcinogen N-[4-(5-Nitro-2-furyl)-2-thiazolyl]acetamide

Don B. Headley, Samuel M. Cohen and George T. Bryan


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/37/4/974

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.