Temporal Comparisons of Immune Status and Target Organ Histology in Mice Fed Carcinogenic 5-Nitrofurans and Their Nornitro Analogs

Don B. Headley, Roger G. Klopp, Preston M. Michie, Erdoğan Ertürk, and George T. Bryan

Division of Clinical Oncology, Department of Human Oncology, Wisconsin Clinical Cancer Center, Madison, Wisconsin 53792

ABSTRACT

Immune status and target organ histology were temporally evaluated in mice fed either carcinogenic 5-nitrofurans or their noncarcinogenic nornitro analogs. Mice were fed either the leukemogen \(N\text{-}[4-(5\text{-nitro-2-furyl})\text{-2-thiazolyl}]\text{acetamide (NFTA)}\) or the urinary bladder carcinogen \(N\text{-}[4-(5\text{-nitro-2-furyl})\text{-2-thiazolyl}]\text{formamide}\) or their nornitro analogs, \(N\text{-}[4-(2\text{-furyl})\text{-2-thiazolyl}]\text{acetamide}\) or \(N\text{-}[4-(2\text{-furyl})\text{-2-thiazolyl}]\text{formamide}\), at three dosage levels for a period of 12 weeks. The antibody-mediated immunity and cell-mediated immunity were evaluated via the Cunningham modification of the Jerne plaque method and cell-mediated lympholysis technique, respectively. NFTA-fed mice exhibited statistically significant and dose-dependent immunosuppression of antibody- and cell-mediated immunities. Antibody-mediated immunity responses at the high dose (1000 ppm) were 19, 19, 31, and 19% of control values at Weeks 4, 7, 10, and 13, respectively. Cell-mediated immunity responses were 29, 25, and 8% of control values at Weeks 7, 10, and 16, respectively (p < 0.01). Mice fed \(N\text{-}[4-(5\text{-nitro-2-furyl})\text{-2-thiazolyl}]\text{formamide}\), \(N\text{-}[4-(2\text{-furyl})\text{-2-thiazolyl}]\text{acetamide}\), or \(N\text{-}[4-(2\text{-furyl})\text{-2-thiazolyl}]\text{formamide}\) showed occasional depressions of immune responses. These responses were unsustained and appeared to be biologically anomalous when compared to responses of NFTA-fed mice. The immunosuppressed state of NFTA-fed mice was observed prior to the histological appearance of leukemia. Leukemia development in NFTA-fed mice was highly dose dependent with the latent period inversely proportional to NFTA dosage. Hyperplastic foci in the thymus, spleen, or lymph nodes were noted early in the feeding period. Leukemic involvement of these organs was observed at later times. Despite the short feeding time, \(N\text{-}[4-(5\text{-nitro-2-furyl})\text{-2-thiazolyl}]\text{formamide-fed mice surviving >18 weeks exhibited a urinary bladder hyperplasia (20 of 85), transitional cell carcinoma (2 of 85), and leukemia (30 of 85). The nornitro analogs, \(N\text{-}[4-(2\text{-furyl})\text{-2-thiazolyl}]\text{acetamide}\) and \(N\text{-}[4-(2\text{-furyl})\text{-2-thiazolyl}]\text{formamide}\), failed to exhibit any statistically significant tumor incidences.

INTRODUCTION

Since the initial report by Malmgren et al. (27) in 1952, many chemical (20, 30, 32, 37, 38), physical (39), and viral (33, 35) oncogens have been identified as immunosuppressants. Some of these agents may suppress only humoral (30, 32) or both humoral and cellular immune responses (20, 31, 37, 38). The question of whether or not immunosuppression is a necessary precondition for neoplastic transformation or proliferation has been addressed in the cases of several carcinogenic hydrocarbons (34, 42). Immunosuppressive oncogens show considerable variation with respect to time, magnitude, and duration of immunosuppression. The correlation and significance between immunosuppressive and oncogenic potential of agents remains uncertain at this time (30).

5-Nitrofurans were extensively studied for carcinogenic potential, and many of these compounds were shown to be carcinogens in several species (3, 6). The carcinogenicity of these compounds was strongly dependent on the presence of the 5-nitro group (1, 5, 14, 15). While the 5-nitrofuran, NFTA, was a powerful leukemogen (4, 7) and immunosuppressant (20, 21) for mice, the urinary bladder carcinogen, FANFT, was a powerful leukemogen (4, 7) and immunosuppressant (20, 21) for mice, the urinary bladder carcinogen, FANFT, despite the close structural similarity to NFTA, has not demonstrated significant immunosuppressive activity (8, 23). These carcinogenic 5-nitrofurans are of close structural similarity but differing target organ specificity. Thus, they provide a unique system with which to study immunological status and histological changes during the neoplastic transformation process.

In this study, we report the temporal relationship of the humoral and cellular immune responses to the pathological changes in BALB/c mice fed the carcinogens NFTA and FANFT and their nornitro analogs FTA and FAFT at 3 dosage levels.

MATERIALS AND METHODS

Chemicals. The structure of the chemicals studied are shown in Chart 1. NFTA was obtained from U. Ravizza (Milan, Italy), and FANFT was from Saber Laboratories, Inc. (Morton Grove, Ill.). The nornitro analog, FTA, was synthesized in our laboratory via previously described methods (22). Chemical identity and purity of all test compounds were checked by melting point and IR spectrophotometry.

FAFT, a new compound, was prepared by dissolving 2-amino-4-(2-furyl)-1,3-thiazole (100 g) in 200 ml of pyridine at 23°; 100 ml of formic acid were then added dropwise (22). The mixture was heated at 110–120° for 2 hr and then cooled in an ice bath. The crystals, collected and recrystallized in ethyl acetate or methanol with a 50% yield of bright yellow prisms, had a melting point of 196–198°. Results of elemental analysis were:

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[1] The abbreviations used are: NFTA, \(N\text{-}[4-(5\text{-nitro-2-furyl})\text{-2-thiazolyl}]\text{acetamide}\); FANFT, \(N\text{-}[4-(5\text{-nitro-2-furyl})\text{-2-thiazolyl}]\text{formamide}\); FTA, \(N\text{-}[4-(2\text{-furyl})\text{-2-thiazolyl}]\text{acetamide}\); FAFT, \(N\text{-}[4-(2\text{-furyl})\text{-2-thiazolyl}]\text{formamide}\); AML, antibody-mediated immunity; PFC, plaque-forming cells; CML, cell-mediated lympholysis; %SR, percentage of specific release; CMI, cell-mediated immunity.
N-[4-(5-nitro-2-furyl)-2-thiazolyl] acetamide
(NFTA)

N-[4-(5-nitro-2-furyl)-2-thiazolyl] formamide
(FANFT)

N-[4-(2-furyl)-2-thiazolyl] acetamide
(FTA)

N-[4-(2-furyl)-2-thiazolyl] formamide
(RAFT)

Chart 1. Structures, chemical names, and assigned abbreviations (in parentheses) of test compounds.

C₆H₁₀O₄N₂S
Calculated: C 49.48, H 3.09, N 14.43
Found: C 49.15, H 3.16, N 14.23

Immunological Assays. AMI was estimated by determining the number of direct PFC per mouse spleen via Cunningham modification of the Jerne plaque method (10, 20). The immunizing dose was 0.1 ml of a 25% packed sheep RBC suspension given 4 days prior to assay. Four replicated slides were made for each mouse.

The CML used was a modification of the method of Thorn et al. (40). BALB/c mice (H-2b) were sensitized with an i.p. injection of 2 x 10⁷ EL4 cells (H-2b) and killed 8 to 10 days later for assay. EL4 target cells were labeled with Na₂⁵¹CrO₄ (200 to 500 Ci/g chromium). The range of labeling of EL4 was 0.1 to 0.5 cpm/cell. Positive controls consisted of EL4 cell-immunized mice receiving no other treatment, and negative controls were nonimmunized mice receiving no other treatment. Spleen cells from the 3 test mice within any one group were pooled, and the viability was determined to be ≥95% as measured by trypsin blue exclusion. Spleen cell to target cell ratios of 10:1, 30:1, and 100:1 were used, and these cell suspensions were incubated for 3 hr at 37° in the wells of a microtiter test plate in replicates of 8. Well samples were counted in a Nuclear Chicago Mark II liquid scintillation counter, optimized for ⁵¹Cr. Total lysis was determined by freeze-thawing a hypotonic suspension of labeled EL4 cells 3 times. %SR was calculated with the formula

\[
\% \text{SR} = \left( \frac{\text{treatment cpm} - \text{negative control cpm}}{\text{total lysis cpm} - \text{negative control cpm}} \right) \times 100
\]

Experimental Design. Female BALB/c mice (Timco, Inc., Houston, Texas) 5 weeks old at the beginning of the experiment were prospectively and randomly allocated to one of 12 experimental groups of 91 mice each and to a control group of 293 mice. The mice were allowed to acclimate for 3 to 6 days before the start of the experiment. Test mice were fed ad libitum diets containing either NFTA or FANFT at doses of 100, 500, or 1000 ppm or FTA or FAFT at doses equimolar to those of NFTA and FANFT, respectively, through the end of the 12th week. The control group received unmedicated pelleted commercial diet (Wayne Lab Blox; Allied Mills, Inc., Chicago, Ill.). Each compound was mixed with ground control diet as described previously (4, 28) and stored at 4°. Three to 5 mice from each group were killed at predetermined times throughout the experiment for temporal immunological assays and carcinogenicity evaluation. Autopsies were performed when possible on all mice that were killed or found dead, and the tissues were sectioned and stained with hematoxylin and eosin for light microscopic examination (4, 28).

Tumor Transplantation. Enlarged thymuses of 4 mice fed NFTA at 1000 ppm were removed for transplantation at Weeks 14 and 15 and from 2 mice at Week 28 and were identified as NFTA-I, NFTA-II, NFTA-III, and NFTA-IV, respectively. Samples of thymuses were saved for histological examination, and the remaining portions were gently dispersed in Eagle's minimal essential medium (Grand Island Biological Co., Grand Island, N. Y.) and injected into syngeneic mice either s.c. into the abdominal wall or i.p.

Statistics. Student's t and \( \chi^2 \) tests were used for determining statistical significance of immunological and carcinogenic data, respectively (16, 36). \( p \) values ≤0.05 were considered statistically significant. S.E. for CML data was estimated through the use of the propagation of errors method (25, 26).

Leukemia and bladder hyperplasia incidences were analyzed with the one-hit model with background

\[
p = 1 - e^{-(BD+A)}
\]

the 2- and 3-hit models with background, and the logistic model

\[
p = \frac{e^{(A+BD)}}{1 + e^{(A+BD)}}
\]
as tests for dose dependency (9, 19), in which \( D \) is dose; \( A \) is 0.0105 and \( B \) is 43.1762 for NFTA leukemias; \( A \) is 0 and \( B \) is 6.5430 for FANFT bladder hyperplasia.

RESULTS

Chemical Ingestion and Animal Growth. Food consumptions and weight gains were similar in all groups. The average weekly consumptions of test compounds were estimated to be 220, 110, and 22 μmol for mice fed high, medium, and low doses, respectively. Although unexpected deaths occurred, these were not limited to any specific group and did not reflect any manifestation of drug toxicity.

NFTA Feeding Results. The immunological and carcinogenic data for NFTA-fed mice are summarized in Chart 2. The number of nucleated spleen cells for control mice remained relatively constant at 3.1 ± 0.3 x 10⁸ spleen cells/spleen over the 18-week test period. NFTA-fed mice demonstrated a direct and consistent relationship between the dose of leukemogen and the degree of AMI immunosuppression as measured by the number of PFC per spleen. PFC per spleen of high-dose-fed mice was 19% of the control value at Week 4 and remained significantly suppressed through Week 16 (4 weeks after cessation of medicated diet) when the number of PFC per spleen was 55% of the control value. The number of nucleated
throughout the 18-week test period for AMI assessment via the Cunningham slide thereaf-er fed unmedicated control diet. Age-matched control mice received only unmedicated control diet ( ). Mice were killed at predetermined times ( ). CMI assessment via the CML using the pooled spleen cells of 3 mice for each time point (B), or carcinogenic evaluation with positive controls for 10: 1, 30:1, and 100:1 spleen cell:target cell ratios were 11.3 ± 6.8, 16.5 ± 7.4, and 27.4 ± 7.9, respectively, over the 18-week test period. The %SR showed the same relationship to NFTA dosage.

Evidence of malignancy resulted in the mouse being scored as tumor-bearing ( ). If all tissue was normal, the mouse was scored as normal ( ). For mice used in CML, the %SR's in positive controls for 10:1, 30:1, and 100:1 spleen cell:target cell ratios were 11.3 ± 6.8, 16.5 ± 7.4, and 27.4 ± 7.9, respectively, over the 18-week test period. The %SR showed the same relationship between test compounds and positive controls at these 3 ratios, and therefore only the 100:1 data are presented. Spleen cells per spleen of test mice was inversely proportional to NFTA dosage.

The number of PFC per 10⁶ spleen cells in mice fed NFTA at 1000 ppm decreased to 33% of the control value at Week 4 and remained suppressed throughout the 18-week test period. This reduction in PFC per 10⁶ spleen cells was also dose dependent. Mice fed NFTA at 1000 ppm had 53% of the control value of the spleen cells at Week 13, while mice fed NFTA at the medium or low doses had normal or slightly elevated numbers of spleen cells, respectively.

For mice used in CML, the %SR's in positive controls for 10:1, 30:1, and 100:1 spleen cell:target cell ratios were 11.3 ± 6.8, 16.5 ± 7.4, and 27.4 ± 7.9, respectively, over the 18-week test period. The %SR showed the same relationship between test compounds and positive controls at these 3 ratios, and therefore only the 100:1 data are presented. Spleen cells of mice fed medium or low doses of test compounds demonstrated an elevated response at early times. There was no consistent pattern of enhanced or suppressed responses during the 18-week test period with the noted exception of NFTA-fed mice. Mice fed NFTA at the medium dose of 500 ppm generally showed responses intermediate to those fed high and low doses. This depressed response was statistically significant at Week 14. Mice fed NFTA at 1000 ppm exhibited suppressed responses of 29, 25, and 8% of control values at Weeks 7, 10, and 16, respectively.

Two leukemias and one lung adenocarcinoma were noted in the 187 histologically examined control mice. In NFTA-fed mice exhibiting lymphoid tissue hyperplasia, the more severe cases occurred in those that were fed higher doses or received NFTA for longer time periods (4, 18). Most mice that were fed the lower doses or lived shorter times microscopically exhibited only hyperplasia of thymuses, spleens, or lymph nodes. Leukemia incidences in mice surviving >18 weeks were 4 of 29, 19 of 28, and 20 of 20 for mice that received NFTA at 100, 500, or 1000 ppm, respectively. The morphology of the lymphocytic leukemia observed was identical to that described by Dunn (12) and reported previously by us (4). Mean latent periods of leukemia detection for these groups were 45 ± 5, 38 ± 2, and 22 ± 2 weeks, respectively. For the NFTA-induced leukemias, the one-hit model with background was the best fit of the statistical models tested. In addition to leukemia, there were lung adenocarcinoma incidences of 4 of 75, 10 of 75, and 1 of 66 and forestomach carcinoma incidences of 0 of 74, 1 of 75, and 2 of 66 for mice fed at 100, 500, and 1000 ppm, respectively. No other tumors were noted.

**FANFT Feeding Results.** The immunological and carcinogenic data for FANFT-fed mice are summarized in Chart 3. No significant change from control values was noted in the number of PFC or nucleated spleen cells of FANFT-fed mice at any dose or time tested. An instance of statistically significant suppression of CML response occurred at Week 10 when mice fed FANFT at 1000 ppm exhibited 27% of the control response. FANFT-fed mice failed to exhibit consistent significant immunosuppression or any dose-related immunological response.

Leukemia incidences of mice fed FANFT at doses of 100, 500, and 1000 ppm were 17 of 80, 6 of 67, and 13 of 71, respectively. Latent periods of FANFT-induced leukemia did not differ from that of control periods (52 ± 2 weeks). Urinary bladder hyperplasia incidences were dose dependent with incidences of 0 of 80, 9 of 67, and 13 of 71 at the low, medium, and high doses, respectively. Two transitional cell urinary bladder carcinomas were observed in the 1000-ppm group. The bladder tumors grew into the submucosa, invading the submucosal and serosal fat tissues (13). Urinary bladder hyperplasia data were consistent with the one-hit-with-back- ground statistical model. Lung adenocarcinoma incidences for FANFT-fed mice in the low-, medium-, and high-dose groups were 1 of 80, 1 of 67, and 3 of 71, respectively. No other tumors were observed.

**FTA Feeding Results.** Mice fed FTA at the high dose had reduced PFC at Week 13. No significant changes from control values in the PFC or CML were noted at other doses or times tested. Numbers of spleen cells did not differ significantly from control values. Lung adenocarcinomas at incidences of 1 of 79, 1 of 83, and 1 of 81 for low, medium, and high doses, respectively, were the only tumors observed.
DISCUSSION

This study indicates that the 5-nitrofururan, NFTA, attacks primarily the lymphoid cells in mice to produce a generalized leukemia and severe and sustained suppression of both AMI and CMI. The immunosuppression was observed well in advance of histological appearance of leukemia. Use of the more sensitive CML permitted an earlier detection of NFTA-induced CMI suppression than reported previously by use of the graft-versus-host assay (20). This study (20) also indicated AMI suppression by the sixth day of NFTA administration. Data presented here show the parallel development of the immunosuppressed state with the pathological changes in lymphoid and other tissues.

The mechanism of immunosuppression which best fits these data for NFTA appears to be the direct effect of the compound itself or more likely an activated biotransformation product on the immunocompetent cells or stem cells prior to and independent of the final neoplastic transformation. Although the sensitivity of the methods used for detecting histological neoplastic change are less than those for detecting immunosuppression, the identifiable leukemic changes appear too long after the observed immunosuppression for the presence of neoplasia to provide a reasonable explanation for this phenomenon. The immunosuppression may be a phenotypically detectable expression of a preneoplastic metabolic change. It is not known whether the immunosuppression resulted from direct genetic interaction with NFTA or if the resultant neoplasia arose from a previously immunosuppressed cell. Suggested hypotheses for the continued suppression of immune function in mice after removal of NFTA from diet may include the result of preneoplastic metabolic alterations and cachectic effects of concomitant tumor growth.

Although immunocompetence could be used as a marker of the functional integrity of lymphoid cells, no similar biochemical characteristic response of bladder epithelial cells could be monitored in FANFT-fed mice. The urinary bladder carcinogen FANFT appears to induce tumors without any sustained systemic immunosuppression. The microenvironement of the urinary bladder has not yet been examined for immunocompetence during the neoplastic process. It is possible that impairment of immunological function in the host may provide a more suitable and hospitable milieu for the development of neoplasia. However, the absence of any sustained immunosuppression by the bladder carcinogen FANFT supports earlier data that its

FAFT Feeding Results. Mice fed FAFT at the high and medium doses had 80% of the number of nucleated spleen cells as age-matched controls throughout the 18-week test period. Cell numbers returned to control values after cessation of medication. PFC were suppressed at Week 4 (23% of the control value), while CML showed suppressed responses of 69 and 34% of control values at Weeks 10 and 16, respectively. Lung adenocarcinoma incidences for FAFT-fed mice in the low-, medium-, and high-dose groups were 2 of 75, 1 of 77, and 1 of 74, respectively. No other tumors were observed in FAFT-fed mice.

Tumor Transplantation. Tissue from enlarged thymuses of 4 mice fed NFTA at 1000 ppm and histologically confirmed to be leukemic were transplanted for 4 to 8 generations. Lowest transplantable cell numbers for tumor establishment in syngeneic hosts were 1 × 10³ for NFTA-I, 5 × 10⁴ for NFTA-II, 1 × 10⁶ for NFTA-III, and 5 × 10⁷ for NFTA-IV. Tumor cells transplanted s.c. into the abdominal wall often invaded the regional lymph nodes and muscle. Tumor cells transplanted i.p. invaded the spleen, thymuses, intestinal submucosa, mesenteric, pararenal, and intestinal lymph nodes. The degree of positional and cytological anaplasia as well as cellular invasion was more extensive in tumors of subsequent transplant generations than in original hosts.
carcinogenicity is independent of any detectable systemic immunosuppressive activity (3, 8, 23).

The fact that many carcinogens are immunosuppressive is not surprising given the direct relationship between chemical carcinogenicity and cytotoxicity (17). The rapid proliferative activity of lymphocytic cells renders them sensitive targets for this cytotoxicity.

The 5-nitro moiety on the furan ring has been shown to be of great importance in the antibacterial, mutagenic, and carcinogenic properties of nitrofurans (1, 11, 24). Carcinogenic 5-nitrofurans have been shown to inhibit DNA, RNA, and protein synthesis in bacteria and also bind to mammalian macromolecules in vivo (2, 29, 41). These properties of macromolecular interaction may provide a suitable explanation for the early suppression of AMI and CMI responses observed here. The nonnitro analogs FTA and FAFT were devoid of any detectable biological activities.

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REFERENCES


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