Thymic lymphomas in Wistar rats exposed to N-methyl-N-nitrosourea (MNU)

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The Brazilian Agency for the Environment (IBAMA) recently adopted an alternative medium-term multiple-organ assay system with the Wistar rat strain for detection of the carcinogenic potential of pesticides. Originally, this initiation-promotion protocol was established in Japan with the isogenic Fischer 344 male rat. Among the initiating agents used in that assay, N-methyl-N-nitrosourea (MNU) rapidly induces malignant lymphoma and leukemia and early mortality of rats from different strains. This study was developed to evaluate whether the outbred Wistar rats are also similarly susceptible to MNU. Particularly, it aimed to evaluate the dose-response relationship and to register the MNU-induced pre-neoplasia and neoplasia that may develop in the lympho-hematopoietic system (LHS) of the Wistar rat within a medium-term period. Four groups of male Wistar rats were treated during 2 weeks with vehicle or with MNU (80, 160 or 240 mg/kg body weight, i.p.). After sacrifice at the 12th and 20th weeks, the thymus, spleen, bone marrow, cervical and mesenteric lymph nodes and liver were collected for analysis. At the 20th week, LHS malignant tumors and benign vascular tumors occurred only in the high- and intermediate-dose MNU-treated animals. Four animals treated with 240 mg/kg developed diffuse thymic lymphomas; two others, treated respectively with 240 mg/kg and 160 mg/kg, developed spleen hemangiomas. The present observations indicate that the Wistar strain is as susceptible as other strains to the early development of MNU-induced LHS (pre)neoplasia. Therefore, this strain seems suitable to be used as test system in bioassay protocols that adopt MNU as an initiating agent for carcinogenesis. (Cancer Sci 2003; 94: 240–243)

The standard experimental procedure for detection of the carcinogenic potential of chemicals is the long-term bioassay with rats and mice.[1, 2] However, due to its operational complexity, long duration and high cost, several alternative protocols have been proposed.[1, 3] Among these, a medium-term (26-weeks) multiple-organ bioassay was established with the male Fischer 344 rats by a group of researchers led by Nobuyuki Ito, at the Nagoya City University Medical School.[4–6] Based on the initiation-promotion concept of chemical carcinogenesis, that bioassay is also referred to as DMBDD bioassay, after the initials of the five genotoxic chemicals used to initiate the carcinogenesis process in multiple target organs: N-diethylnitrosamine (DEN), N-methyl-N-nitrosourea (MNU), N-butyl-N-(4-hydroxybutyl)nitrosamine (BBN), N,N′-dimethylhydrazine (DMH) and N-bis(2-hydroxypropyl)nitrosamine (DHPN).[4–6] In Brazil, the Institute for the Environment and Natural Resources (IBAMA) adopted in 1996 a modified protocol of the DMBDD bioassay as a valid source of evidence of the carcinogenic potential of pesticides.[7] This modified protocol uses Wistar rats of both sexes instead of the male Fischer 344 rat. Since then, our laboratory has conducted several studies to build up a catalog of the lesions induced by the initiating agents in the Wistar rat.[8, 9]

Among the initiating agents used in the DMBDD bioassay, MNU has a broad spectrum of target organs, including particularly the lympho-hematopoietic system (LHS).[10] In fact, when provided at relatively high doses, MNU characteristically induces early and high incidence of leukemia and malignant lymphomas in young rats,[10–12] resulting in a high mortality rate of the animals and limiting the development of long-term experimental studies. In the present study the LHS was chosen for evaluation because it is an early target of MNU and its morphologic evaluation may provide rapid evidence of the carcinogenic influence of that agent in the Wistar strain.

In the original DMBDD bioassay with the Fischer 344 male rat, MNU is applied at a sub-carcinogenic dose level (80 mg/kg).[5, 6] The mortality rate and the spectrum of LHS (pre)neoplasias at a relatively low MNU dose level have not been established for the Wistar rat in medium-term observation. Therefore, a dose-response evaluation of MNU-dependent characteristic lesions—such as early LHS tumors—is a necessary step to better establish the DMBDD assay with the Wistar strain.

In this 20-week study we examined morphologically the LHS of male Wistar rats exposed to three different doses of MNU. Since the bone marrow, the lymph nodes, the spleen and the thymus should be considered as a single system,[13] we performed an integrated, simultaneous morphologic evaluation of these organs.

Materials and Methods

Animals. Eight-week-old male Wistar rats from a local strain were obtained from the Paraná Institute of Technology (TEC-PAR, Curitiba, Brazil) and housed five per polypropylene plastic cage with wood chips for bedding in an animal room with controlled conditions of temperature (22±2°C), humidity (55±10%) and lighting (12 h light/dark). They were fed *ad libitum* commercial chow (NUVILAB-CR1, NUVITAL, Paraná, Brazil) and tap water. The experiment started after an acclimation period of 4 weeks. The University Committee for Ethics in Animal Research approved the procedures adopted in this study (Protocol No. 119/00).

Chemical carcinogen. N-Methyl-N-nitrosourea (MNU, Sigma Chemical Co., St. Louis, MO, Catalog No. 684-93-5) was used to initiate the carcinogenesis process.

Experimental design. The experimental design is presented in Fig. 1. Male rats of similar body weights (220–230 g) at the beginning of the experiment were allocated to four experimental groups, one control (untreated) and three groups which received, during the first 2 weeks of the experiment, four i.p. injections of MNU (two injections/week) at the following doses: 20 mg/kg of body weight (total of 80 mg/kg), 40 mg/kg (total of 160 mg/kg) or 60 mg/kg (total of 240 mg/kg). After carcinogen treatment, they were kept on basal diet and water *ad libitum* until sacrifice at the 12th and 20th weeks of the experiment, when they were killed by exsanguination under ether an-

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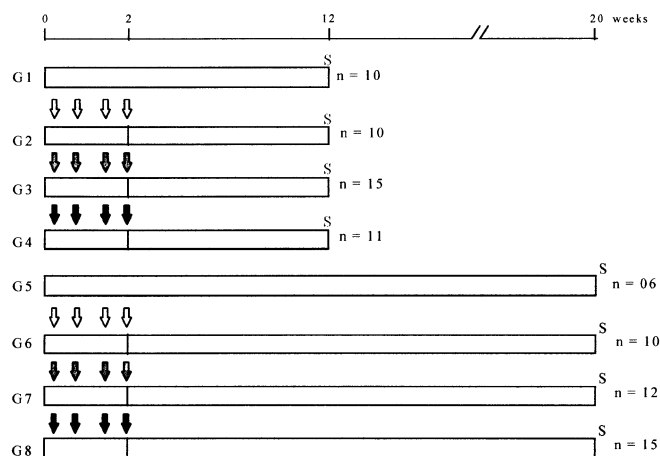
Fig. 1. Experimental design. MNU=N-methyl-N-nitrosourea. ⇩ MNU 80 mg/kg i.p. (4×20 mg), ⬇ MNU 160 mg/kg i.p. (4×40 mg), ⬇ MNU 240 mg/kg i.p. (4×60 mg). *n*, effective number of animals; S, sacrifice.

esthesia. Complete gross examination was performed for detection of tumor masses. The thymus, spleen, bone marrow (right femur), lymph nodes (cervical and mesenteric) and liver were collected for histological analysis. The liver and spleen were weighed immediately after removed and blotted dry. The 20th week of the study was established as the latest moment for observation, taking in consideration the information that MNU at 160 mg/kg of body weight i.p. induces leukemia and malignant lymphoma in 10–20% of exposed male Fischer 344 rats before this time point.[10, 14] Accordingly, the 12th week was proposed as a convenient point for detection of possible preneoplastic lesions.

Histological and immunohistochemical analysis. The liver, spleen, thymus, lymph nodes, the right femur and any tumor mass found were fixed in 10% buffered formalin during 48 h. After fixation, the right femur was decalcified, washed in tap water and sectioned. Paraffin-embedded sections (5 μm thickness) were stained with hematoxylin and eosin (H&E) for histological analysis. Sections of a lymphoma found in the mediastinum of one animal were submitted to immunohistochemical staining with anti-cytokeratin antibodies (M3515, DAKO Co., Carpinteria, CA) for identification of epithelial remnants within the tumoral mass to confirm the thymic origin of the neoplasia. Sections of the liver were also submitted to immunohistochemical detection of putative preneoplastic foci of hepatocytes that express the enzyme glutathione S-transferase, placental form (GST-P) with anti-GST-P antibodies (MBL, Nagoya). The immunohistochemical studies were performed using the avidin-biotin method.[15] The anti-cytokeratin and the anti-GST-P antibodies were used at the concentrations of 1:300 and 1:1000, respectively.

Hematological evaluation and bone marrow cytology. During necropsies, peripheral blood samples were collected in vacutainer tubes with 15% EDTA and differentially quantified through a T890 Coulter counter for the following: leukocyte, erythrocyte and platelet counts, hematocrit, hemoglobin concentration, mean corpuscular volume, mean corpuscular hemoglobin and mean corpuscular hemoglobin concentration. Cytological smears of the left femur bone marrow were prepared immediately after necropsies and stained by Leishman's method.[16]

Statistical analysis. Analysis of variance (ANOVA) was used to evaluate the differences among the body weights and the absolute and relative weights of liver and spleen of the groups treated with the four different doses of MNU. The differences

of incidence of lesions among the groups were assessed by applying the Goodman test.[17] Differences were considered significant when $P<0.05$.

Results

Body weights. The lower (80 mg/kg) and intermediate (160 mg/kg) MNU-dosed groups gained body weight progressively during the experiment, and at the end of the study (20th week) they did not differ significantly from the control group. At this time point, however, the highest dose group (240 mg/kg) presented a mean body weight significantly lower (−17%) than the control.

Lympho-hematopoietic organs. At the 20th week, the mean relative spleen weight of the highest dose group was significantly increased (+21%) when compared to the other groups. However, no conspicuous spleen histological alteration was apparent in these animals. An animal treated with 240 mg/kg presented a large mediastinal lymphoma, which diffusely infiltrated the thoracic wall and the diaphragm, involving the liver and the spleen. Under H&E staining, this was a medium-sized cell type thymic lymphoma[18] composed predominantly of monotonous sheets of lymphoblastic cells and a few macrophages which gave the appearance, at low magnification, of a "starry sky" pattern (Fig. 2). The thymic origin of this lymphoma was confirmed by the demonstration of cytokeratin-positive probable remnants of the thymic Hassal's bodies within the tumor mass (Fig. 3). No other gross alteration of the thymus was found in any animal in the experiment.

Other malignant and benign tumors were found histologically in the LHS in the high- and intermediate-dose MNU-treated animals (Table 1). Three incipient thymic lymphomas occurred in the animals treated with 240 mg/kg of MNU; they were unilateral intralobular neoplasias that did not enlarge the organ (Fig. 4). Two animals, treated respectively with 240 mg/kg and 160 mg/kg, developed spleen hemangiomas and one animal treated with 160 mg/kg presented spleen angioectasias.

At both 12th and 20th weeks, the hematological parameters were within the range of reference values for control rats.[19] Also, no animal in this study presented conspicuous histological alterations in the lymph nodes or in the bone marrow of the right femur. Accordingly, no alterations in the cytological pattern of the left femur bone marrow smears were verified in any group at the 12th and 20th weeks. The bone marrow smears showed intact and high cellularity of the granulocytic, erythro-
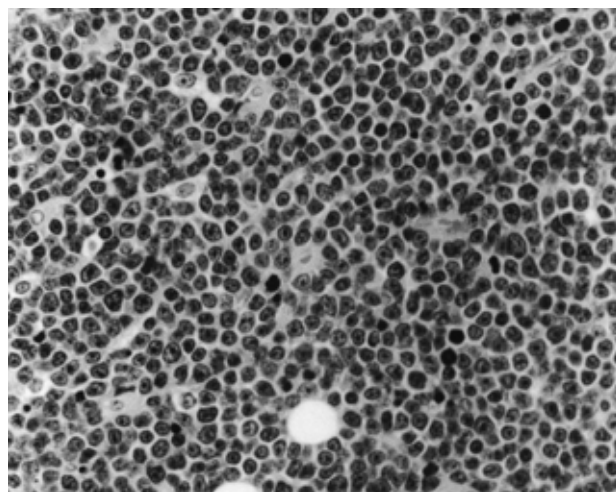
Fig. 2. Diffuse lymphoblastic thymic lymphoma (medium-sized cells type). (H&E, 1000×)
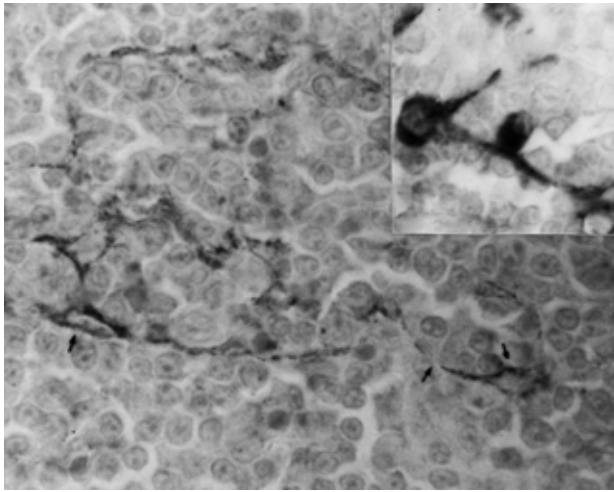
Fig. 3. Diffuse lymphoblastic thymic lymphoma—probable remnants of thymic Hassall's epithelial bodies within the lymphoma: cells and reticular structures positive for AE-1/AE3 cytokeratins. (Immunostaining by the avidin-biotin peroxidase method, 1000×)
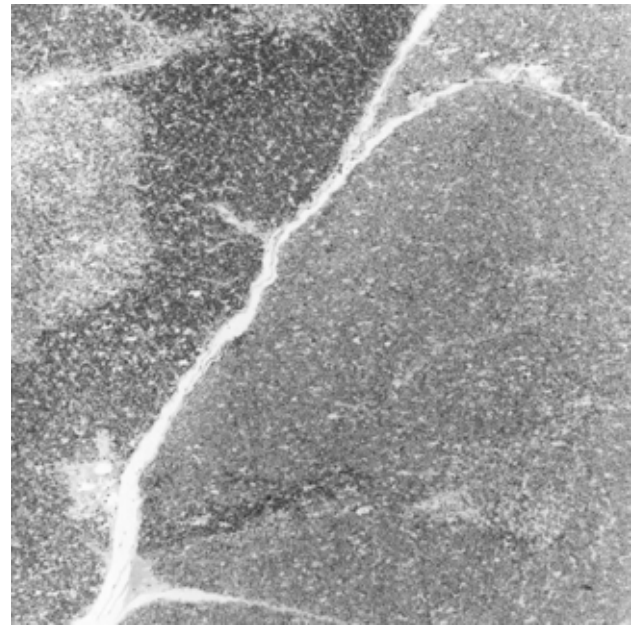
Fig. 4. Thymus lobule (at right) altered by diffuse lymphoma growth. The lobule at the left retains the normal thymic structure. (H&E, 132×)

Table 1. Incidences of (pre)neoplasia in the thymus and spleen of male Wistar rats at the end of the experiment (20th week)

Organ/lesion	Experimental groups[1]			
Effective number of animals	G_0	G_{80}	G_{160}	G_{240}
	6	10	10 (%)	15 (%)
Thymus				
Lymphoma	0	0	0	4 (27)
Spleen				
Angiectasia	0	0	0	1 (7)
Hemangioma	0	0	1 (10)	1 (7)
Liver				
GST-P⁺ foci[2]	0	0	0	15 (100)[3]

1) G_0, G_{80}, G_{160} and G_{240}=control and MNU-treated animals with 80, 160 and 240 mg/kg, respectively.
2) GST-P⁺ foci=foci of altered hepatocytes expressing the enzyme glutathione S-transferase, placental form.
3) A few foci with 3–4 positive hepatocytes each.

cytic and megakaryocytic cell lineages irrespective of whether the animal was treated with MNU or not.

Liver. At the 20th week, the mean absolute and relative liver weights did not differ among the groups, irrespective of MNU treatment. Although no conspicuous histological alterations were seen in the liver of any group, every animal exposed to 240 mg/kg of MNU showed a few preneoplastic GST-P-positive minifoci in the liver, with 3–4 hepatocytes each.

Discussion

Putative MNU-related effects were verified only at the 20th week of the study in the animals exposed to the highest dose (240 mg/kg), as shown by significantly diminished mean body weight and tumor development in the thymus and spleen. The 12th week seemed to be too early for detection of MNU-related LHS alterations; preneoplastic incipient lesions were registered only at the 20th week, synchronously with well-established thymus and spleen neoplasia. As will be discussed below, these early lesions corresponded to intra-lobular thymic lymphoma, spleen angioectasias and GST-P⁺-altered foci of hepatocytes, which should be considered as putative markers of the MNU-initiated carcinogenesis in the Wistar rat.

The thymic lymphomas found in this study at a 27% incidence should be assumed as dependent on MNU, because these tumors did not occur in control rats or in intermediate- and lower-dose treated animals, and the rats that developed tumors were younger than 28 weeks of age, when spontaneous tumors are not common in this species.[20] In one animal the neoplasia was very aggressive and infiltrated thoracic and abdominal organs, but in three other rats, the microscopically diagnosed thymic lymphomas showed only intra-lobar involvement, i.e., they were in a very early stage of development. These neoplastic growths were lymphoblastic lymphomas, like the early MNU-induced thymic lymphomas described in other rat strains.[18, 21]

The incidence of thymic lymphomas induced by nitrosoureas in rats has been reported as variable, depending on the strain and on the duration of the study. Fischer 344 rats treated with a total dose of 240 mg/kg of MNU—the same as the highest dose of the present study—for 6 weeks and sacrificed 18 weeks later presented a 97% incidence of thymic lymphomas.[12] Sprague-Dawley animals treated intra-gastrically with 360 mg/kg of MNU showed a 100% incidence of thymic lymphoma at the 15th week.[21] After treatment with 400 μg/ml of propylnitrosourea (PNU) dissolved in drinking water for 28 weeks, the incidence of thymic lymphomas was different among Fischer 344 (incidence of 98%), Wistar/Furth (71%), Sprague-Dawley (29%), ACI/Ms (23%), Donryu (24%) and Long-Evans (10%) rats.[22] Therefore, although relatively low, the 27% incidence of thymic lymphoma observed in the highest-dose group at the 18th week after the end of the MNU-treatment points to a medium-term carcinogenic influence of that chemical in the Wistar strain.

MNU acts as a direct carcinogenic alkylating agent, and persistent O⁶-methylguanine adducts have been proposed to be the critical lesion in a variety of MNU-induced tumors. Tissues more susceptible to the carcinogenic effect of nitrosoureas have 3 to 18 times less activity of the DNA repair enzyme alkyltransferase than tissues less susceptible to these agents.[23–25] When transgenic mice express the human O⁶-alkylguanine-DNA alkyltransferase in the thymus they present a reduced incidence (from 58% to 4%) of thymic lymphomas after MNU exposure, that is, the thymus is protected from developing neo-

plasia.[23] Therefore, the susceptibility of the rodent thymus to develop earlier and more aggressive MNU-dependent tumors seems to depend particularly on its limited capability for DNA repair through the enzyme O^6-alkylguanine-DNA alkyltransferase.[23, 26]

Since MNU has a broad spectrum of target organs, the possibility exists that if the experiment had been extended in time, tumors would have developed in other tissues.[10, 26] The present study, however, focused only on LHS tumors because they could be used as early markers of the carcinogenic influence of MNU on the Wistar strain. The development of putatively preneoplastic liver GST-P$^+$ foci of hepatocytes was also evaluated, because this has been an important parameter in the rat alternative medium-term bioassays for carcinogenesis.[5, 27] In the present case, GST-P$^+$ foci were associated only with the highest dose of MNU, being rare and very small, in concordance with the weak MNU initiation activity in the liver.[28]

The low total incidence (2/25, 8%) of spleen hemangiomas in the intermediate and high-dose treated groups makes it difficult to establish a causal relationship of these benign lesions with MNU treatment. However, since spleen hemangiomas occurred only in MNU-treated animals and are reported to be of very rare occurrence in young rats,[29] it seems likely that they were also dependent on MNU exposure. The finding of angioectasia in the spleen of another animal also exposed to the intermediate dose of MNU supports the possibility that the spleen vessels are also a target of the carcinogen toxicity. In this context, it can be assumed that spleen vascular ectasia is a preneoplastic lesion of hemangiomas, as proposed by others.[30]

The rapid development of thymus and spleen (pre)neoplasia after exposure to a relatively high dose level of MNU indicates that the outbred Wistar male rat is as susceptible as other rat strains to the carcinogenic influence of that carcinogen, developing marker lesions in the lympho-hematopoietic system that can be used as end-points in alternative carcinogenesis protocols.

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