Natural Killer Activity in a Medium-term Multi-organ Bioassay for Carcinogenesis

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Natural killer (NK) cell activity was evaluated after the initiation and promotion steps in a medium-term multi-organ bioassay for carcinogenesis. NK cell activity was assessed in vitro by Cr51 release assay at the 4th and 30th weeks of the experiment. Male Wistar rats were sequentially initiated with N-diethylnitrosamine (DEN i.p.), N-butyl-N-(4-hydroxybutyl)nitrosamine (BBN drinking water), N-methyl-N-nitrosourea (MNU i.p.), dihydroxy-di-N-propylnitrosamine (DHPN drinking water) and N,N′-dimethylhydrazine (DMH s.c.) at subcarcinogenic doses for 4 weeks (DMBDD initiation). One group was evaluated at the 4th week and the other was maintained without any further treatment until the 30th week. Two initiated groups were exposed through the diet to 2-acetylaminofluorene (2-AAF) or phenobarbital (PB), from the 6th until the 30th week. Five additional groups were studied to evaluate the effects of each initiator on NK activity. All groups submitted to initiation only, initiation plus promotion, or promotion only, developed significantly more preneoplastic lesions than the untreated control group. The main target organs for tumor development in the initiated animals were the liver and the colon, irrespective of treatment with 2-AAF or PB. NK cell activity was not affected by exposure to genotoxic carcinogens after initiation, at the 4th week. Treatments only with PB or 2-AAF did not change NK cell activity. However, decreased NK cell activity was registered in the group only initiated with DMBDD and in the group given DMBDD+2-AAF. This late depression of NK cell activity at the 30th week could be related to the production of suppressing molecules by the tumor cells.

Key words: NK cell activity — Immune response — Multi-organ carcinogenesis — Chemical carcinogens

Several chemicals have been shown to affect adversely natural killer cell activity in vivo and in vitro3-5 and to influence the immune competence in man and laboratory animals.4, 5 Depression of immune function induced by these agents could serve as a stimulus for carcinogenicity, by allowing transformed cells to bypass the normal host immune surveillance.3, 6 On the other hand, an agent capable of enhancing natural killer (NK) cell activity may raise the host’s resistance to tumor neoantigens.3 A number of experimental studies have demonstrated that NK cells can impair the growth and metastasis formation of a variety of transplantable tumors.7, 8 Regarding chemical carcinogens, there is evidence that the incidence of certain chemically induced tumors is increased in immunosuppressed mice.9 Activated NK cells can destroy initiated stem cells in a murine intestinal carcinogenesis model, preventing the establishment of their progeny and thereby eliminating the potential development of neoplasia.10, 11

Studies on the carcinogenic potential of chemicals have been carried out with different experimental protocols.12, 13 An alternative multi-organ system has been proposed using wide-spectrum initiation involving several organs.12, 14 This bioassay protocol is based on the initiation-promotion concept of carcinogenesis. It consists in a sequential treatment with five potent carcinogens permitting a multi-organ initiation and an exposure to the test substance in order to evaluate its promoting potential.12, 14, 15 The advantages of this model include the relatively short experimental period, low cost, and an increased sensitivity for the detection of chemical promoters of carcinogenesis.12 Recently, the medium-term multi-organ bioassay for carcinogenesis (DMBDD) was officially adopted in Brazil as a source of evidence of the carcinogenic potential of chemicals.10 Since the protocol consists of two operationally distinct steps for initiation and promotion, it conveniently allows the study of the immune system participation at each one of these steps of the carcinogenic process.

In the present study we investigated the NK cell activity in Wistar rats after the steps of initiation and promo-
tion in a medium-term multi-organ bioassay using genotoxic and non-genotoxic chemical carcinogens.

MATERIALS AND METHODS

Animals A total of 117 male Wistar rats were obtained from the Paraná Institute of Technology (TECPAR, Curitiba, Brazil). They did not receive any treatment prior to the study. At the beginning of the acclimation period they were 4 weeks old. The rats were randomly distributed in polypropylene cages covered with metallic grids. All animals were supplied with filtered water in 500 ml clear glass bottles with rubber stoppers and stainless steel controlled-flow sipper tubes. They were fed with NUVILAB-CR1 (NUVITAL, Curitiba, Brazil) ad libitum.

Room temperature was set and controlled at 22°C, humidity at 55% and the lighting consisted of alternate 12 h light and dark cycles. Animal body weights were registered weekly during the first 4 weeks and then at every 4 weeks until the end of the experiment. Water and food consumptions were measured weekly during initiation and every 15 days from weeks 6 to 30.

Chemical agents N-Diethylnitrosamine (DEN), N-methyl-N-nitrosourea (MNU), N,N'-dimethylhydrazine (DMH) and 2-acetylaminofluorene (2-AAF) were purchased from Sigma Chemical Co. (St. Louis, MO); N-butyl-N-(4-hydroxybutyl)nitrosamine (BBN) and phenobarbital (PB) were purchased from Tokyo Kasei Industries Co. (Tokyo) and dihydroxy-di-N-propylnitrosamine (DHPN) was from Nacalai Tesque, Inc. (Kyoto).

Experimental design The experimental design is presented in Fig. 1. Thirteen groups were composed as follows. Two groups (1a, 1b), killed at the end of 4th and 30th weeks respectively, were used as untreated controls. Four groups (2a, 2b, 3a, and 3b) were treated sequentially at sub-carcinogenic doses with five initiating agents (DMBDD treatment). DEN (100 mg/kg body wt., i.p., single dose at the commencement), MNU (20 mg/kg body wt., i.p., 4 times, 2 doses a week) and BBN (0.05% in drinking water during 2 weeks) were given during the 1st and 2nd weeks. During the 3rd and 4th weeks these groups were treated with DMH (40 mg/kg body wt., s.c., 4 times, two doses a week) and DHPN (0.1% in drinking water during 2 weeks). One of these four groups (group 2a) was killed at the end of the 4th week and another (group 2b) was maintained without any further treatment until the 30th week. Two other initiated groups (groups 3a and 3b) were supplied with 2-AAF (0.01%) or PB (0.05%) mixed in the diet, from the 6th until the 30th week. Two non-initiated groups (groups 4a and 4b) received only 2-AAF or PB through the diet.

Five additional groups (groups 5 to 9) were studied to evaluate separately the effects of the chemical initiators, DEN, BBN, MNU, DHPN and DMH (Fig. 1). These groups were killed at the end of the 4th week and compared to the groups treated with the complete set of carcinogens (DMBDD, group 2a) and to the respective untreated control (group 1a).

Histological analysis Complete necropsies were performed on the animals at the 30th week. All gross lesions including tumors were recorded. Target organs such as liver, lung, esophagus, stomach, and small and large intestine, kidneys, urinary bladder, lymph nodes, thymus and spleen, were removed, fixed in 10% buffered formalin, processed and stained with hematoxylin and eosin for microscopic examination. Before fixation, the spleen was cut into two sections, one of which was used fresh for NK cell activity assay.

51Cr release cytotoxicity assay NK cell activity was determined using a modification of a previously described 51Cr release assay. Spleen pieces were disaggregated into RPMI-1640 (Cultilab, Campinas, Brazil) culture medium supplemented with gentamycin 20 mg/ml (Sigma), glutamine (Gibco, Gaithersburg, MD) and 10% inactivated fetal calf serum (Cultilab). Mononuclear spleen cells were separated by Histopaque (d=1.083 g/liter) (Sigma) gradient centrifugation, washed with RPMI and incubated at 37°C, 5% CO₂ for 1 h on Petri dishes for depletion of adherent cells. The resulting non-adherent cells were adjusted to 5×10⁶ cells/ml and 100 µl of both effector cells (splenic non-adherent cells) and target cells (⁵¹Cr-labeled YAC-1 adjusted to 1×10⁵ cells/ml) were added in triplicate to round-bottomed microtiter plates (Corning Coster Co., Acton, MA) for dilution. The effec-
tor-target ratios were 50:1, 25:1, 12.5:1 and 6.25:1. Spontaneous isotope release was measured following culture of YAC-1 alone, and maximum release by the addition of Triton X-100. The plates were incubated at 37°C, 5% CO₂, for 4 h and after this 100 µl of supernatant from each well was carefully removed and counted in a γ counter (Gamma Nuclear, Budapest, Hungary). Specific lytic activity (%) was calculated as follows:

\[
\% \text{ Specific lytic activity} = \frac{\text{experimental release} - \text{spontaneous release}}{\text{maximum release} - \text{spontaneous release}} \times 100
\]

**Statistical analysis** Data on body weight gain were analyzed by analysis of variance (ANOVA) and the effects of the treatments on NK cell activity (%) were analyzed for 50:1 effector:target ratio using the Kruskal-Wallis test at \( P<0.01 \). The number of neoplasias per group was evaluated by the Kruskal-Wallis test with the significance level set at \( P<0.05 \). The number of tumor-bearing rats and the number of rats with preneoplastic lesions were evaluated by the Fisher and the \( \chi^2 \) tests, at significance levels of \( P<0.05 \) and \( P<0.001 \).

**RESULTS**

**Body weights** The animals of the different groups adjusted well to the experimental conditions. At the end of the 4th week, the mean body weight gain of group 2a, which received the complete DMBDD treatment, was significantly \( (P<0.05) \) lower than that of the untreated control group (group 1a) (data not shown). However, at the end of the 30th week, the mean body weight gain of the initiated only group had returned to the control level. Also, at the end of the experiment, the group treated with DMBDD+2-AAF (group 3a) presented a significantly \( (P<0.01) \) diminished body weight gain when compared to group 4a, which received only 2-AAF, or to the control 1b group (data not shown).

**NK cell activity** Rats exposed to each one of the 5 initiators (DEN, BBN, MNU, DHPN or DMH) or to their com-

![Fig. 2. NK cell activity at the end of 4th week after treatment with the initiators. □, control; ▲, DMBDD; △, DEN; ●, BBN; ◊, MNU; ○, DHPN; ■, DMH.](image)

![Fig. 3. NK cell activity at the 30th week after treatments with 2-AAF and phenobarbital. (A) 2-AAF. □, control; ▲, DMBDD; ◊, DMBDD+2-AAF; ●, 2-AAF. (B) Phenobarbital. □, control; ▲, DMBDD; ◊, DMBDD+PB; ●, PB. Statistical analysis for \( P<0.05 \): *significantly different from control group; **significantly different from DMBDD group.](image)
A combination (DMBDD) did not exhibit changes in the NK cell cytotoxicity against YAC-1 tumor cells at the end of 4th week (Fig. 2). At the 30th week, the NK cell activity in the groups treated only with 2-AAF or PB (groups 4a or 4b) was not different from that in the control group 1b (Fig. 3, A and B). However, the NK cell activity in group 2b, which received DMBDD treatment, and in group 3a, treated with DMBDD + 2-AAF, was significantly decreased (Fig. 3A) when compared to that of the control group 1b (P < 0.01). The NK cell activity in group 3b, treated with DMBDD + PB, was significantly higher than that of group 2b, which received only the DMBDD initiation (P < 0.01) (Fig. 3B).

**Morphologic analysis** The incidence and distribution of putative preneoplastic and neoplastic lesions in the different groups are shown in Table I. Prenecoplastic lesions were found in the liver (altered foci of hepatocytes), urinary bladder (simple hyperplasia and papillary and nodular hyperplasia), colon (aberrant crypt foci) and kidneys (altered tubular cell foci) (Table I). Neoplastic lesions were found in the groups submitted to initiation, followed or not by promotion. They were observed mainly in the liver and in the colon (Table I). All groups submitted to initiation only, initiation plus promotion, or promotion only, presented a higher number of preneoplastic lesions when compared to the untreated control group (group 1b) (P < 0.001) (Table II). The number of benign tumors per group was significantly higher in the groups treated with DMBDD + 2-AAF (group 3a) and DMBDD + PB (group 3b), when compared to group 2b, treated only with DMBDD (P < 0.05) (Table II). In the group treated with DMBDD + 2-AAF (group 3a), both the number of malignant tumors and the number of tumor-bearing rats were significantly higher than in the groups treated with

| Effective number of animals: | DMBDD | DMBDD + 2-AAF | DMBDD + PB | Control | 2-AAF | PB |
|-------------------------------|-------|---------------|------------|--------|-------|----|
| Small intestine               | 14    | 9             | 11         | 5      | 8     | 9  |
| Adenocarcinoma                | 0     | 1 (11)        | 0          | 0      | 0     | 0  |
| Colon                         | 14    | 9             | 12         | 5      | 8     | 9  |
| Aberrant crypt foci           | 1 (7) | 1 (11)        | 0          | 0      | 0     | 0  |
| Adenoma                       | 1 (7) | 0             | 1 (8)      | 0      | 0     | 0  |
| Adenocarcinoma                | 4 (28)| 6 (66)        | 2 (18)     | 0      | 0     | 0  |
| Kidneys                       | 28    | 18            | 22         | 10     | 16    | 18 |
| Clear cell foci               | 0     | 1 (5)         | 0          | 0      | 0     | 0  |
| Eosinophilic cell foci        | 0     | 0             | 1 (4)      | 0      | 0     | 0  |
| Basophilic cell foci          | 1 (3) | 4 (22)        | 0          | 0      | 0     | 1 (5) |
| Tubular adenoma               | 0     | 0             | 2 (9)      | 0      | 0     | 0  |
| RMTa                          | 0     | 1 (5)         | 0          | 0      | 0     | 0  |
| Urinary Bladder               | 13    | 9             | 11         | 4      | 8     | 9  |
| Simple hyperplasia            | 3 (23)| 7 (78)        | 6 (54)     | 0      | 3 (37)| 2 (22) |
| PN hyperplasiab               | 0     | 3 (33)        | 3 (27)     | 0      | 1 (12)| 0  |
| Papilloma                     | 0     | 0             | 0          | 0      | 0     | 0  |
| Carcinoma                     | 0     | 1 (11)        | 0          | 0      | 0     | 0  |
| Liver                         | 14    | 9             | 11         | 5      | 8     | 9  |
| Clear cell foci               | 14 (100)| 7 (78)       | 9 (75)     | 0      | 8 (100)| 1 (11) |
| Eosinophilic cell foci        | 12 (86)| 7 (78)       | 9 (75)     | 0      | 8 (100)| 2 (22) |
| Basophilic cell foci          | 7 (50)| 5 (42)        | 4 (33)     | 0      | 4 (50)| 0  |
| Amphophilic cell foci         | 5 (36)| 7 (78)        | 3 (17)     | 0      | 2 (22)| 0  |
| Adenoma                       | 0     | 2 (22)        | 2 (17)     | 0      | 1 (12)| 0  |
| Cholangioma                   | 0     | 2 (22)        | 0          | 0      | 0     | 0  |
| Hepatocellular carcinoma      | 0     | 2 (22)        | 1 (8)      | 0      | 0     | 0  |
| Cholangiocarcinoma            | 0     | 3 (33)        | 0          | 0      | 0     | 0  |

Table I. Incidence of Preneoplastic and Neoplastic Lesions at the 30th Week

- a) RMT: renal mesenchymal tumor.
- b) PN: papillary or nodular hyperplasia.
- c) Number of organs analyzed.
- d) Percentage of animals with neoplasia.
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Table II. Incidence of Preneoplastic and Neoplastic Lesions and Tumor Burden at the End of the 30th Week

| Groups   | Treatment          | Effective number of animals | Number of animals with preneoplastic lesions (%) | Benign tumors | Malignant tumors |
|----------|--------------------|-----------------------------|------------------------------------------------|---------------|-----------------|
|          |                    |                             |                                                 | Number of tumor-bearing rats (%) | Number of tumors/group | Number of tumor-bearing rats (%) | Number of tumors/group |
| Initiated|                    |                             |                                                 |               |                 |                              |                       |
| 2b       | DMBDD              | 14                          | 14° (100)                                      | 1 (7)         | 1               | 4 (28)                       | 4                       |
| 3a       | DMBDD+2-AAF        | 9                           | 9° (100)                                       | 4 (44)        | 4°              | 7 (78)°,***                  | 14°,***                |
| 3b       | DMBDD+PB          | 12                          | 12° (100)                                      | 5 (42)        | 5°              | 3 (25)°                      | 3                       |
| Non-initiated|                |                             |                                                 |               |                 |                              |                       |
| 1b       | Control            | 5                           | 0                                              | 0             | 0               | 0                            | 0                       |
| 4a       | 2′-AAF            | 8                           | 8° (100)                                       | 1 (12)        | 1               | 0                            | 0                       |
| 4b       | PB                | 9                           | 5° (55)                                        | 0             | 0               | 0                            | 0                       |

a) (DEN+BBN+MNU+DHPN+DMH).
b) DMBDD+2-acetylaminofluorene.
c) DMBDD+phenobarbital.
d) 2-Acetylaminofluorene.
e) Phenobarbital.

Statistical analysis for P<0.05: *significantly different from group 1b; **significantly different from group 2b; ***significantly different from group 3b.

DISCUSSION

Animals treated with the DMBDD protocol for multi-organ carcinogenesis developed preneoplastic lesions and tumors, respectively, in the liver and in the colon (Table I). The number of tumor-bearing rats and the number of malignant neoplasias were significantly higher in the group treated with DMBDD+2-AAF than in the DMBDD and DMBDD+PB treated groups (Table II). Animals exposed only to 2-AAF (group 4a) did not develop any malignant tumor in the same period. These results indicate that the initiation of carcinogenesis was accomplished by the DMBDD treatment and that 2-AAF can exert an enhancing influence on the carcinogenic process, as previously registered with the same DMBDD protocol.18) The present two-step model of chemical carcinogenesis allowed us to evaluate NK cell activity both after initiation (when DNA damage occurs) and after promotion (when proliferation of altered clones gives rise to preneoplastic and neoplastic lesions).19, 20)

Our results on NK cell activity at the end of the 4th week, just after the treatment with the initiators, did not show any change in cytotoxicity, as assessed by the ⁵¹Cr release cytotoxicity spleen assay. This indicates that NK cell activity was not affected by the toxicity of the five genotoxic initiators. However, the alkylating agents used, DEN, BBN or MNU could have induced early and/or transient alterations of NK cells, which rapidly returned to normal, since immune responses can be restored within 2 weeks after treatment.21) The present data are in agreement with those of Locniskar et al.,22) who used a different experimental protocol. They reported that the NK cell activity of Fischer rats was not altered in a model of colon carcinogenesis induced by DMH after 1 week, 2 months or 5 months of treatment. Talcott et al.3) observed that NK cell activity decreased in a dose-related manner in DEN-treated rats. In another study, MNU did not exert any consistent toxic effect on NK cell activity during the early stages of tumor development.23) Thus, a direct relationship between chemical carcinogen administration and early immunosuppression, as measured by NK cell activity, appears to be of questionable significance for the future development of tumors.

The NK cell activity at the 30th week was significantly decreased in the group submitted only to the initiators (DMBDD) and in the DMBDD+2-AAF group. Neither 2-AAF nor PB administered to non-initiated animals significantly affected NK cell activity. These results suggest that the lower NK cell activity seen in initiated animals could be related to some long-term suppressing effect of the DMBDD initiation on NK cell activity. The DMBDD+PB group presented higher NK cell activity than the DMBDD group, and the possibility exists that the non-genotoxic agent PB induced tumors biologically distinct from those induced by 2-AAF in the initiated group. Indeed, benign lesions were found mainly in the DMBDD+PB group, while malignant tumors predominated in the DMBDD+2-AAF group. It is possible that tumors occurring in these different groups could induce different kinds of cytokines.24) A comparative study between 2-AAF and 4-AAF suggested that chemicals without genotoxic/carcinogenic
activity in rats, such as 4-AAF, generally fail to influence NK cell activity.25,26

The present findings of deficient NK cell activity in the initiated group at the 30th week, are in agreement with those reported previously by Hong et al.26 In a different multi-organ carcinogenesis model, these authors showed that NK cell activity in rats treated with carcinogens was not different from the control in the early stage of carcinogenesis (4th, 10th and 20th weeks), but was suppressed at the 30th and 40th weeks. Thus, the occurrence of malignant tumors seems to have some influence on NK cell activity, since in the present study the group which developed more malignant tumors (group 3a) presented decreased NK cell activity.

Deficient NK cell activity is commonly found in cases of advanced cancer. This has been noted in patients with advanced cervical cancer, possibly as a consequence of tumor invasion, which results in NK cell depression.27,28 The inhibition of NK cell activity could be related to the production of cell growth-related or other molecules by the tumor cells.29 Recent studies have shown that NK cell-mediated lysis may be regulated by loss or reduced expression of MHC class I molecules by the tumor cells.30,31 Several lines of evidence have indicated that the expression of certain major histocompatibility complex (MHC) molecules could inhibit NK cell activity at the target cell level.32 Some tumors, mainly colon tumors, can lose the expression of one of these MHC class I molecules.30,32 This phenomenon inhibits the recognition of the tumor cells by T CD8+ lymphocytes and confers resistance to the attack of NK cells, allowing in vivo selective advantages.30,32 This resistance of tumor cells to the attack by the NK cells could be related to the expression of receptors known in the murine system as NKR-P1 and Ly-49.34,35 The NKR-P1 molecule is expressed on all NK cells and may play a role in triggering NK cytotoxicity. Conversely, the Ly-49 receptors that recognize class I MHC molecules are expressed on subsets of NK cells and inhibit NK-mediated lysis.36,37 It is also possible that NK-mediated lysis would be negatively regulated not only by the expression of MHC class I molecules, but also by non-MHC molecules known as Cho-1 antigens. Indeed, some tumors can produce interferon-γ (IFN-γ) and stimulate NK cell activity.7 On the other hand, target cells in the presence of the IFN-γ may express Cho-1 antigens on the cell surface, thus conferring resistance to NK cell-induced lysis of target cells.29 NK cell activity may be also inhibited by indirect factors such as prostaglandins, and adherent or non-adherent cells, which negatively regulate NK cell activity and suppressor cytokines in rodents.7

In summary, our results show that the initiation procedure of a medium-term multi-organ protocol with five different genotoxic chemicals does not affect the NK cell activity as seen at the 4th week of the experiment. A 25-week-long dietary exposure to PB or to 2-AAF also did not change the NK cell activity. At the end of the 30th week, however, the NK cell activity was diminished in the group submitted to the initiation procedure and also in the DMBDD+2-AAF group. At this time, several animals in these groups presented benign and malignant tumors. The depression of NK cell activity could be related to the production of suppressing molecules by the tumor cells.29 Further studies should be done to provide better information on the depression of NK cell activity in the advanced stage of carcinogenesis.

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