

Effect of 20-methylcholanthrene on amphibian natural killer cells

I. A. Sadek^{1,*}, M. Ghoneum², E. L. Cooper³

¹ Department of Zoology, Faculty of Science, University of Alexandria, Egypt

² Department of Otolaryngology, Charles R. Drew University of Medicine and Science, Los Angeles, California, USA

³ Department of Anatomy, UCLA School of Medicine, Los Angeles, California, USA

ABSTRACT Effects of the chemical carcinogen 20-methylcholanthrene (MC) on toad *Bufo marinus* natural killer (NK) cells were tested *in vivo* and *in vitro*. Toads fed with MC (5 mg toad⁻¹ once per wk) mixed with olive oil exhibited an initial increase in NK activity at 10 d, followed by a suppression (60 %) at 20 and 30 d compared to control toads fed only olive oil. Conjugate formation was examined and demonstrated a slight decrease. Leukocytes which were preincubated with MC (5 to 100 µg ml⁻¹) at room temperature for 1 h, and afterwards washed 3 times, had lowered NK activity (47 to 85 %). In conclusion, the observed inhibition of NK cell activity by MC may predispose toads to carcinogenesis by a mechanism similar to that which occurs in mammals.

INTRODUCTION

Amphibian tumors have been studied by several workers (Khudoley & Picard 1980, Sadek 1981, 1986a). More recently, it has been reported that chemical carcinogens induced tumors in the Egyptian toad (Sadek 1986b, Sadek & Abdelmeguid 1986). Multiple effects of chemical carcinogens on various homeostatic regulatory mechanisms, including the immune system, have been demonstrated during the extremely complicated process of carcinogenesis (Kalland 1984). The immune surveillance theory implies that chemical carcinogens would suppress certain immunocompetent cells, enabling cells with malignant potential, which would otherwise be eliminated, to survive (Herberman 1984).

The idea of immune surveillance has gained renewed interest with the discovery of natural killer (NK) cells which do not require prior sensitization in order to destroy certain tumor cells. Accumulating evidence supports the assumption that NK cells may be part of the natural defense against tumors (Herberman 1983). A decrease in activity of NK cells has been reported in animals treated with chemical carcinogens such as methylcholanthrene (MC) (Ghoneum et al. 1987b), dimethylbenzanthracene (DMBA) (Ehrlich et al. 1983) and urethane (Gorelick & Herberman 1981). In addition, MC is a potent inhibitor of mouse NK activity *in vitro* (Kalland 1984).

Clearly, amphibians have a well-developed immune system that consists of major cellular and humoral components (Cooper 1986). Recently, our studies showed that the immune system of frogs possesses inducible killer (IK) cells (CTL-like) and spontaneous (SK) cells (NK-like) which effect lytic responses against allogeneic cells (Ghoneum & Cooper 1987, Ghoneum et al. 1987a). In this paper, we report on studies aimed at analysing the effects of MC on NK activity *in vivo* and *in vitro* in the toad *Bufo marinus*. Our eventual goal is to examine the evolutionary basis of immune responses towards MC challenge.

MATERIALS AND METHODS

Animals. Sexually mature male and female toads *Bufo marinus*, were used in this study. The toads were obtained from St. Croix Biological, Stillwater, Minnesota, USA. They were kept in plastic pans with water which was changed twice weekly. The average weight per experimental toad was 270 g. Toads were fed meal worms twice per week when the water was changed, and acclimated to the laboratory for 2 wk prior to the experiments.

Preparation of peripheral blood leukocytes (PBL). Toads were sacrificed after anesthetization with ether. Heparinized peripheral blood was obtained by cardiac puncture. The blood was centrifuged in Wintrobe tubes, the buffy coat was carefully removed, washed

* Addressee for correspondence

twice in Amphibian Ringer Solution (ARS) and finally effector cells were adjusted to 10×10^6 cells ml^{-1} (Ghoneum & Cooper 1987, Ghoneum et al. 1987a).

Target cells. The YAC-1 tumor cell line, a Maloney Leukemia virus-induced mouse T-cell lymphoma of A/Sn mouse origin, was maintained in our laboratory in complete medium (CM) at a starting concentration of 3×10^5 cells ml^{-1} .

Complete medium (CM). CM consisted of RPMI-1640, supplemented with 10 % heat-inactivated fetal calf serum (FCS) and 1 % antibiotics ($100 \mu\text{g ml}^{-1}$ penicillin and $100 \mu\text{g ml}^{-1}$ streptomycin).

Methylcholanthrene. 20-methylcholanthrene (MC) was purchased from Sigma Chemical Company, St. Louis, Michigan, USA. For *in vivo* experiments, MC was dissolved in olive oil (Sigma Chemical Company, St. Louis, Michigan, USA). Experimental toads were fed (by esophageal tubing) 5 mg toad^{-1} of MC dissolved in 0.1 ml of olive oil once per wk for 30 d. Control toads were fed 0.1 ml olive oil once per wk. For *in vitro* experiments, MC was diluted in buffer and dissolved in dimethylsulfoxide (DMSO) at concentrations of 5, 20 and $100 \mu\text{g ml}^{-1}$ in $5 \mu\text{l}$ DMSO. Leukocytes were preincubated with MC at room temperature for 1 h.

Cytotoxic assay. YAC-1 target cells (5×10^6 cells) cultured for 24 h were labelled with $100 \mu\text{Ci}$ sodium chromate solution (New England Nuclear, Boston, Massachusetts, USA) for 1 h in 0.1 ml CM and washed 3 times in 5 ml CM Hanks' balanced salt solution (HBSS) as previously described by Ghoneum et al. 1987b. Target cells (1×10^4) in 0.1 ml CM were pipetted into 96 well-round bottom Linbro plates (Linbro Chemical Co., Hamden, Connecticut, USA). Effector cells were suspended at $10 \times 10^6 \text{ ml ARS}$, and 0.1 ml pipetted into quadruplicate wells to give effector:target (E:T) cell ratios of 100:1, 50:1 and 25:1. Following 4 h at room temperature, plates were centrifuged at 1400 rpm ($400 \times g$) for 5 min, and the supernatant 0.1 ml from each well was collected and counted in triplicate in a Gamma counter. The percentage of isotope released was calculated using the following formula:

$$\% \text{ lysis} = \frac{\text{Counts min}^{-1} \text{ exptl rel.} - \text{Counts min}^{-1} \text{ sp. rel.}}{\text{Counts min}^{-1} \text{ max. rel.} - \text{Counts min}^{-1} \text{ sp. rel.}} \times 100$$

Spontaneous release (sp. rel.) from the target cells was always 8 to 10 % of total release. Maximum release (max. rel.), was measured by adding 0.1 ml Triton X100 to designated wells. Percent target cell lysis for different E:T ratios used to construct effector titration.

Conjugate formation. Leukocytes (1×10^5) were incubated with 1×10^6 YAC-1 target cells in 1 ml of ARS in $12 \times 75 \text{ mm}$ glass tubes, sedimented by centrifugation at $130 \times g$ for 5 min and incubated for 1 h at 4°C . Pellets were resuspended and cytocentrifuged

smears stained with Giemsa. The percent of conjugates were examined by counting 200 lymphocytes (bound and free) in triplicate samples (Kumagai et al. 1982).

Lytic units (LU). LUs were calculated from effector titration curves according to our previously demonstrated procedure (Ghoneum et al. 1987b). One LU was defined as the number of effector cells required to achieve 10 % lysis. $\text{LU}/10^7$ is the number of LUs in 10 million effector cells.

Statistical analysis. A 2-tailed Student's t-test was used to determine the significance of differences between different experimental groups.

RESULTS

An initial increase in NK activity in MC-treated toads was observed. However, a statistically significant depression of NK activity occurred at subsequent time periods. MC-fed toads revealed a 15, 40 and 45 % decrease in NK activity at 20 d for effector:target (E:T) ratios of 25:1, 50:1 and 100:1 respectively. This suppression became more intensified at 30 d, and showed 85, 68 and 52 % at 25:1, 50:1 and 100:1 respectively (Table 1).

MC induced inhibition of NK activity *in vitro* (Table 2). Leukocytes which were pre-incubated with MC at room temperature for 1 h, and washed 3 times afterwards with ARS, exhibited inhibition of NK activity (47 to 85 %) at 5 to $100 \mu\text{g ml}^{-1}$. MC-treated toads revealed no statistically significant change in conjugate formation when compared to control toads (Fig. 1a). Similarly, MC did not interfere with binding capacity of NK cells when cultured with PBL *in vitro* for 1 h (Fig. 1b).

Table 1. *Bufo marinus*. *In vivo* effect of MC on NK activity in toads. Data are means \pm SD of 5 different toads in each group

Days post-MC treatment	% of cytotoxicity ^a			LU
	25:1 ^b	50:1 ^b	100:1 ^b	
Control	4.6 ± 1.1	9.8 ± 2.1	18.6 ± 3.7	22.2
10	10.3 ± 4.3	16.9 ± 4.6	23.6 ± 3.4	40
20	3.9 ± 2.8	5.9 ± 2.7	10.2 ± 2.8	9.2*
30	0.7 ± 0.3	3.1 ± 1.1	8.9 ± 2.5	6.7**

^a Cytotoxicity assay is a 4 h ^{51}Cr -release assay
^b 25:1, 50:1 and 100:1 representing dilution steps of effector to target cells
* $p < 0.05$; ** $p < 0.001$

DISCUSSION

The attention of some tumor immunologists has recently focused on natural immunity as a possible first line of defense against transformed cells progressing toward malignancy (Herberman 1983, Ghoneum et al. 1987b). Immunosuppression induced in experimental animals by chemical carcinogens such as methylchoanthrene is thought to play a major role in malignant tumor development in these hosts (Herberman 1982). In this work, we wanted to test the effect of MC on NK cell activity in ectothermic vertebrates (e.g. toads) in order to compare this effect with mammals. The results clearly show that MC suppresses NK activity in toads *in vivo* and *in vitro*.

The mechanism of suppression in NK activity induced by MC is generally unknown and according to

Table 2. *Bufo marinus*. *In vivo* effect of MC on toad NK activity. Data are means \pm SD of 5 different toads in each group

Dose ($\mu\text{g ml}^{-1}$)	% of cytotoxicity ^a			LU
	25:1 ^b	50:1 ^b	100:1 ^b	
0	4.6 ± 1.1	9.8 ± 2.1	18.8 ± 3.7	22.2
5	3.0 ± 0.9	5.2 ± 1.6	12.0 ± 3.6	11.8*
20	2.0 ± 0.5	4.6 ± 1.5	5.0 ± 1.2	6.9**
100	1.0 ± 0.4	1.2 ± 0.6	3 ± 1.0	3.3**

^{a,b} As in Table 1
* $p < 0.05$; ** $p < 0.001$

Herberman (1983), may be due to interference with effector mechanisms by such chemical carcinogens. Previous studies suggest that environmental chemicals and tumor promoters interfere with T-cell function and NK activity, which may facilitate tumor growth (Dean et al. 1982). It has been shown that the polycyclic aromatic hydrocarbons (PAH) alter T-cell function and induce suppressor T-cells (Alfred et al. 1982, Wojdani et al. 1984). We have previously detected inhibition in NK activity in C3H mice as early as 10 d post-treatment with MC (Ghoneum et al. 1987b); the decrease in NK activity may be due to non-specific toxic effects of MC on NK cells. Additionally, another PAH compound, DMBA, has been demonstrated to have an inhibitory effect on murine NK activity (Ehrlich et al. 1983), and MC suppressive effects on NK cells *in vitro* has been reported (Kalland 1984). In conclusion, MC inhibits the lytic activity of NK cells in toads against YAC-1 target cells. The latter conclusion may clarify the process of carcinogenesis of MC in Egyptian toads (Sadek 1981, Sadek & Abdelmeguid 1986), which appears to be similar to mammals. Future experiments in this laboratory will attempt to define the mechanism of action of MC on cell-mediated immune (CMI) function and its relationship to immunosurveillance in toads. This will hopefully provide a better understanding of the evolutionary basis of immune responses to tumor growth.

Acknowledgement. This work was partially supported by Fulbright Grant No. 253.

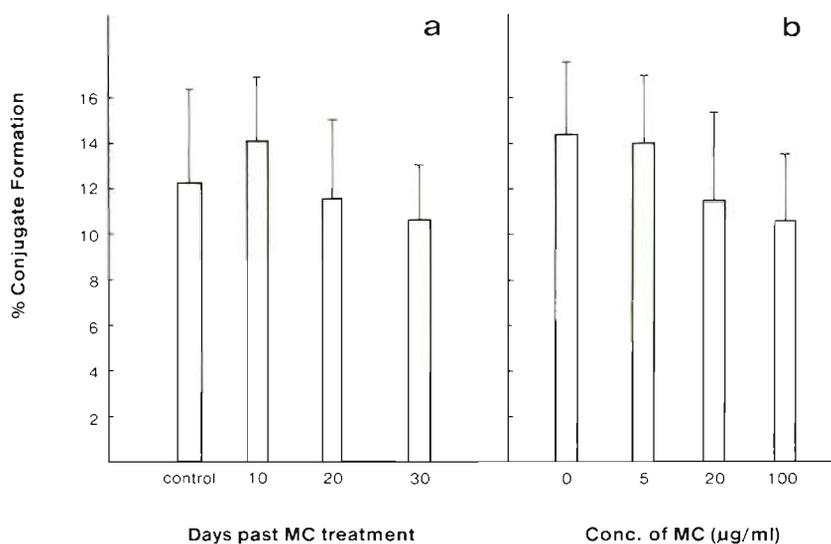


Fig. 1. *Bufo marinus*. Effect of MC on conjugate formation between NK and YAC-1 cells (a) *in vivo* and (b) *in vitro*

LITERATURE CITED

- Alfred, L. J., Wojdani, A., Neito, M., Perez, R., Yoshida, G. (1982). A chemical carcinogen, 3-methylcholanthrene, alters T-cell function and induces T-suppressor cells in a mouse model system. *Immunology* 50: 207-214
- Cooper, E. L. (1976). Immunity mechanisms. In: Lofts, B. (ed.). *Physiology of Amphibia*, Vol. 3. Academic Press, New York, p. 164-272
- Dean, J. H., Luster, M. I., Boorman, G. A., Lauer, L. D., Ward, E. C. (1982). Immunotoxicity of tumor promoting environmental chemicals and phorbol diesters. In: Hadden, J. W., Chedid, L., Dukor, P., Spreafico, F., Willoughby, D. (eds.) *Advances in immunopharmacology*. Pergamon Press, Oxford, p. 23-31
- Ehrlich, R., Efrati, M., Malatsky, E., Shochat, L., Bar-Eyal, A., Witz, I. P. (1983). Natural host of defense during oncogenesis: NK activity on dimethylbenzanthracene carcinogenesis. *Int. J. Cancer* 31: 67-73
- Ghoneum, M., Cooper, E. (1987). Inhibition of frog SK effector: target cell binding. *Dev. Comp. Immunol.* 11: 167-179
- Ghoneum, M., Cooper, E., Smith, C. (1987a). Factors affecting inhibition of spontaneous cytotoxic cells in frogs. *Dev. Comp. Immunol.* 11: 363-373
- Ghoneum, M., Gill, G., Wojdani, A., Payne, C., Alfred, L. J. (1987b). Suppression of basal and *Corynebacterium parvum*-augmented NK activity during chemically induced tumor development. *Int. J. Immunopharm.* 9: 71-78
- Gorelick, E., Herberman, R. B. (1981). Inhibition of the activity of mouse NK cells by urethane. *J. Natl. Cancer Inst.* 66: 543-548
- Herberman, R. B. (1983). Natural killer cells and tumor immunity. *Surv. Immun. Res.* 2: 306-308
- Herberman, R. B. (1984). Possible role of natural killer cells and other effector cells in immune surveillance against cancer. *J. Invest. Dermatol.* 83: 1375-1405
- Kalland, T. (1984). The effect of 3-methylcholanthrene on mouse natural killer cells *in vitro*. *Int. J. Immunopharmac.* 6: 299-305
- Khudoley, V. V., Picard, J. J. (1980). Liver and kidney tumors induced by N-nitroso-dimethylamine in *Xenopus borealis* (Parke). *Int. J. Cancer* 25: 679-683
- Kumagai, K., Itoh, K., Suzuki, R., Hinuma, S., Sitoh, F. (1982). Studies of murine large granular lymphocytes. I. Identification as effector cells in NK and K cytotoxicities. *J. Immunol.* 129: 388-394
- Sadek, I. A. (1981). Vitamin A and its inhibitory effect as tested on Egyptian toads. *Oncology* 38: 23-26
- Sadek, I. A. (1986a). Effect of cholic on tumor in Egyptian toad. *Oncology* 43: 268-270
- Sadek, I. A. (1986b). The Egyptian toad as a sensitive model to show the effect of corn oil on liver tumor induced by DMBA. *Nutr. Res.* 6: 333-335
- Sadek, I. A., Abdelmeguid, N. (1986). Enhancement of liver tumor in the Egyptian toad by oral contraceptive steroids. *J. Nutr. Growth and Cancer* 3: 239-244
- Wojdani, A., Attarzadeh, M., Wolde-Tsadik, G., Alfred, L. J. (1984). Immunotoxicity effects of polycyclic aromatic hydrocarbons on mouse lymphocytes. *Toxicology* 31: 181-189