Exp Oncol 2006
28, 4, 314–318

Experimental
ONCOLOGY

SENSITIVITY OF HUMAN LYMPHOCYTES TO GENOTOXIC EFFECT OF N-METHYL-N-NITROSOUREA: POSSIBLE RELATION TO GYNECOLOGICAL CANCERS

V.A. Tronov[1, *], *I.I. Kramarenko*[1], *A.D. Kozlova*[1], *L.J. Dederer*[2], *A.G. Kedrova*[3], *L.B. Gorbacheva*[2]

[1]*N.N. Semenov Institute of Chemical Physics RAS, Moscow 119991, Russia*

[2]*N.M. Emanuel Institute of Biochemical Physics RAS, Moscow 119991, Russia*

[3]*N.N. Blokhin Cancer Research Center RAMS, Moscow 115478, Russia*

The aim **of this work is to study responses of PHA-stimulated and resting lymphocytes to methylating agent N-methyl-N-nitrosourea (MNU) and to compare sensitivity to this agent of healthy donor lymphocytes and lymphocytes from patients with gynecological cancers.** ***Methods:*** **Cytotoxicity of MNU, apoptotic death of lymphocytes, was evaluated using two common tests — annexin V-FITC detection assay and live/dead double staining assay (nuclear morphological changes). Genotoxic effect of the agent was determined as delayed (secondary) DNA double strand breaks (DSBs) using neutral comet assay both conventional variant and modified for detection of bromodeoxyuridine-labelled comets, produced by proliferating lymphocytes only.** ***Results:*** **Unstimulated lymphocytes were tolerant to geno- and cytotoxic effects of MNU. In contrast to resting cells, proliferating lymphocytes showed significant genotoxicity ($p = 0.0054$) of MNU followed by increased apoptotic death of cells ($p < 0.05$). Average number of secondary DSBs induced by MNU in lymphocytes from patients with gynecological cancers was about 4-fold less than that of lymphocytes of healthy donors. While lymphocytes from cancer patients did not change proliferative index in response to MNU, the agent decreased 2-fold proliferative indices of stimulated lymphocytes from healthy donors.** ***Conclusion:*** **There is a reverse association between geno- and cytotoxicity of MNU in stimulated lymphocytes and the presence of tumor. The relationship appears to be based on MMR-insufficiency in lymphocytes of the cancer patients.**

Key Words: **gynecological cancer, human lymphocytes, mismatch repair (MMR), methylating agents, DNA double strand breaks.**

There is an increasing evidence that deficiency of DNA repair is one of the factor of carcinogenesis. Assessment of total repair capability is now widespread phenotypical marker for predisposition to cancer. It is based on *in vitro* use of challenge mutagen to unmask latent genetic instability and repair insufficiency. Two widely used methods for detecting genomic instability in lymphocytes are applied — the mutagen-sensitivity assay and the comet assay [1–3]. Five well known mechanisms of DNA repair may have a different contribution to total repair capability — direct enzymatic inactivation of DNA damage; nucleotide excision repair (NER), base excision repair (BER), mismatch repair (MMR) and double strand breaks repair.

Gynecological cancers (endometrial, ovarian and uterine cancers) is the leading cause of cancer death among women [4, also see www.cancer.org/statistics/]. Endometrial and ovarian cancers are included in so called hereditary nonpolyposis colon cancer spectrum (HNPCC spectrum) which is characterized by MMR deficiency [5, 6]. In turn, MMR unsufficiency has been shown to be associated with tolerance of cells to some chemical substances especially to alkylating agents [7]. This tolerance appears to be expressed as resistance to genotoxic effect of the agents [8]. Therefore genotoxic resistance to alkylating agents may be a predictive of low level of MMR activity, chemoresistance and aggressiveness of cancer.

Early we have shown that human colorectal tumor cells were resistant to genotoxic effect of methylating agent N-metyl-N-nitrosourea (MNU) [9]. In this study we utilized an *in vitro* mutagen sensitivity assay which used MNU as the challenge mutagen and neutral comet assay to estimate DNA double strand breaks 24 h after pulse-treatment of cells with the agent. Here using this approach we aimed to study responses of stimulated and resting lymphocytes to methylating agent MNU and to compare sensitivity to this agent of healthy donor lymphocytes and lymphocytes derived from patients with gynecological cancer.

MATERIALS AND METHODS

Patients and lymphocyte isolation and treatment. Venous blood samples were obtained from healthy donors and patients with gynecological cancer. All patient gave informed written concent. Mean age of donors (n = 9, females) was 36 years (range: 29–43 years). All studied patients (n = 10, females) were cured in the Department of Gynecology (N.N. Blokhin Cancer Research Center, Moscow). Mean age of patients was 55 years ranging from 32 to 71 years (ovarian cancer, n = 6, and uterine cancer, n = 4).

Blood samples were taken before any therapy. Lymphocytes were isolated by centrifugation (800 g, 30 min, at RT) of heparinized samples of blood (5–10 ml) through a cashion of ficoll-urografine solution with the density of 1.077 g/ml. Pellets of cells were washed twice in PBS and suspended in RPMI-1640 supplemented with L-glutamine (3 mg/ml), 20% of fetal bovine serum, antibiotics and 10 µg/ml phytohe-

Received: October 25, 2006.
*Correspondence: Fax: (495) 6512191
E-mail: tronov@center.chph.ras.ru

Abbreviations used: BER – base excision repair; DSB – double strand break; FITC – fluorescein isothiocyanate; HNPCC – hereditary nonpolyposis colon cancer; MMR – mismatch repair; MNU – N-methyl-N-nitrosourea; NER – nucleotide excision repair, PHA – phytohemagglutinin.

magglutinin (Gibco_BRL, USA). Suspensions (5 ml in sterile centrifuge tubes, 1 × 10^6/tube) were incubated for 24 h in air atmosphere containing 5% CO_2, at 37 °C. All experiments were performed in parallel on stimulated and nonstimulated lymphocytes. MNU (obtained from the Institute of Organic Chemistry, Moscow, Russia) was dissolved in distilled H_2O just before addition to cells. Before MNU treatment lymphocytes were incubated 1 h with 20 µM O^6-benzylguanine (O^6-bzG, Sigma). This concentration was 2-fold higher of that completely inhibiting O^6-methylguanine-transferase (MGMT) in human tumor cells [9]. BrUdr (as stock solution in dimethyl sulfoxide, 3.1 mg/ml) was added simultaneously with MNU up to the final concentration of 100 µg/ml. Cells were not washed from the drugs during 24 h incubation at 37 °C.

Quantification of apoptosis. The fraction of apoptotic cells was measured with two assays representing early and late (executive) phases of the apoptotic response on genotoxic drug treatment: Annexin V-FITC Detection kit (R & D Systems GmbH, Germany), allowing detection with fluorescent microscopy of cell surface changes in apoptotic cells; live/dead double staining kit, allowing to detect the cells with chromatin condensation and nucleus fragmentation. Cells were stained with mixture of two dyes, acridine orange and propidium iodide, and visualized in fluorescent microscope. Both assays in detailes were the same as in protocols for the kits (http://www.merckbiosciences.co.uk/product/QIA76).

Comet assay. Neutral comet assay was performed as previously described [10] with some modifications made to detect DNA-comets contaning BrUdr. In short, 100 µl of cells (10^6 cells per ml) in PBS were mixed with 200 µl of 1% low melting point agarose at 37 °C. 70 µl of the mixture were carefully layered onto microscope slide, covered with cover glass (size 24 x 24 mm and allowed to solidify on an ice-cold surface. The slides were placed in a lysis solution (2.5 M LiCl, 30 mM EDTA, 10 mM Tris-HCl, 0.1% Li-dodecylsulfate, 0.03 mg/ml proteinase K, pH 8.0). After incubation at 37 °C for 14–18 h the lysis solution was replaced by buffer (2.5 M NaCl, 0.1 M EDTA, 10 mM Tris-HCl, 1% Triton-X100, 10% dimethyl sulfoxide, pH 10) for at least 3 h at 8 °C. Slides were placed in a horizontal electrophoresis tank filled with fresh TAE buffer, pH 8.3 and left to equilibrate for 40 min before electrophoresis. Electrophoresis was conducted for 30 min at 0.5 V/cm. After electrophoresis, the slides were placed in alkaline solution (0.3 M NaOH, 1 mM EDTA, pH 13) for 10 min at 8 °C. The slides were flooded with three changes of neutralization buffer (0.4 M Tris, pH 7.4), followed by one wash in PBS, and drained with paper. For localization of BrUdr incorporated in DNA-comets, 25 µl/gel of mouse monoclonal anti-BrdUrd antibody (10 µg/ml; Boehringer Mannheim) were added, covered with coverslips (18 x 18 mm) and incubated for 1 h in dark at RT. After removal of the coverslips, the primary antibody was gently washed off with three changes of PBS and one wash with PBS + 0.1% BSA before addition of 25 µl/gel of secondary antibody (5 µg/ml sheep anti-mouse IgG, fluorescein conjugated; Boehringer Mannheim), which was incubated and washed off as before. Slides were dried on air, dehydrated with methanol and kept in dark at RT for at least 1 month without any changes in fluorescence. For image analysis, slides were counterstained with propidium iodide 1 µg/ml of antifade and visualized in fluorescent microscope (Micromed, LOMO, Russia), with excitation at the wave length of 480 nm, barrier filter 535 nm. Densitometric and geometric parameters of each comet were calculated with comet analysis program CASP [11]. Propidium iodide stained comets were visualized using excitation the wave length of 540 nm and emission the wave length of 635 nm. Olive tail moments (mt) were calculated for ⩾120 comets on each slide. Presented numbers of MNU-induced DNA double strand breaks was calculated by subtracting comet tail moment for untreated cells from the same for MNU-treated ones. Mean difference was calibrated using gamma-irradiation of resting human lymphocytes (°C) with doses 0–40 Gy and yield of double strand breaks to be 50 per diploid genome within this range of doses [12].

Index of proliferation. Fraction of proliferating lymphocytes after 48 h stimulation was determined as the fraction of BrUdr-positive comets after BrUdr incorporation in DNA during 24 h incubation with cells. General number of comets was counted using fluorescence of PI-stained comets.

Statistics. Statistical analysis was performed using STATISTICA, version 5.0 software. Significance of differences between case and control parameters was calculated using a Mann — Whitney's U-test and considering $p < 0.05$ as indicator of significant differences.

RESULTS AND DISCUSSION

Cytotoxic and genotoxic effects of MNU on resting and proliferating lymphocytes. In this study we aim to compare the responses of human lymphocytes to short-term MNU treatment, implying lymphocytes of healthy donors as a control and lymphocytes from cancer patients as a case. These responses include activation of 2 excision repair pathways — base excision repair (BER) and mismatch repair (MMR) [13]. To discriminate contributions of each pathway in the responses, we compared geno- and cytotoxicity of MNU on resting and proliferating (PHA stimulated) lymphocytes. For measurement of DNA damage in proliferating cells we vizualised anti-BrUdr antibody-FITC labelled comets (Fig. 1, *b*) and for resting lymphocytes we counted propidium iodide stained comets (Fig. 1, *a*).

Fig. 2 summarizes the results of detection of DSB (*a, d*) and apoptosis (*b, c, e*, and *f*) in stimulated and unstimulated lymphocytes in response to MNU. The first finding following these data is that unstimulated lymphocytes are tolerant to geno- and cytotoxic action of MNU in the used concentration (see Fig. 2, *d–f*): exposure to MNU (500 µM) had little or no effect on resting peripheral blood lymphocytes. In contrast to

resting cells, proliferating lymphocytes show significant genotoxicity of MNU followed by increased apoptotic death of cells (see Fig. 2, *a–c*). It means that BER and nucleotide excision repair are unlikely to be involved in delayed geno- and cytotoxic effects of MNU in stimulated lymphocytes. This conclusion is supported by the data obtained with gamma-irradiated human lymphocytes [14]: no difference in the rate of DNA repair capacity after irradiation between resting and proliferating peripheral blood lymphocytes was observed. Moreover, the observed delay of toxic effect of MNU may reflect its association with postreplicative DNA repair, MMR, rather than with BER and NER mechanisms. The second finding illustrated by Fig. 2 is that the time of appearance of DNA double strand breaks coincides with the time of appearance of annexin V positive lymphocytes, the sign of early phase of apoptotic death (see Fig. 2, *a* and *b*). In addition to this, morphological markers of apoptosis in stimulated lymphocytes are displayed 24 h later DNA breaks appearance (see Fig. 2, *a vs c*). This indicates DNA double strand breaks (DSBs) to be an initiating signal for apoptosis induced by MNU. The breaks occured prior to apoptosis and likely to be a result of functioning of active MMR mechanism [13, 15]. MNU, monofunctional S_N1 methylating agent, induces two main adducts in DNA N-methylated bases and O^6-methylated guanine (O^6-meG) [16, 17]. The first is repaired by BER pathway prior to replication [18]. The later is repaired by O^6-methylguanine DNA-methyltransferase (O^6MGMT) and by MMR pathway. As MGMT is inhibited by including in the culture of O^6-benzylguanine, O^6-meG is repaired by postreplication MMR mechanism only [19]. MMR-processing of O^6-meG results in single strand gap opposide the modified guanine. Double strand break (secondary DSB) may be formed either due to endonuclease attack or during the following cycle of replication. Therefore we conclude that the secondary DNA breaks (observed 24 h after MNU treatment of lymphocytes) may be an indicator of functional activity of MMR in stimulated lymphocytes. This conclusion is supported by the results of our previous study reported a correlation between number of secondary MNU-induced DSBs and MMR activity in 3 tumor cell lines [9].

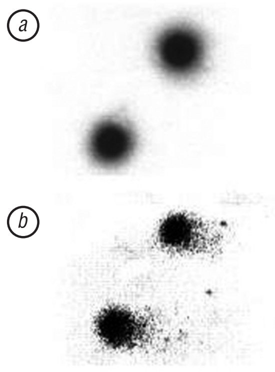

Fig. 1. DNA-comets stained with propidium iodide (*a*) and comets produced by BrUdr-labelled cells and stained with FITC-conugated anti-BrUdr monoclonal antibodies (*b*)

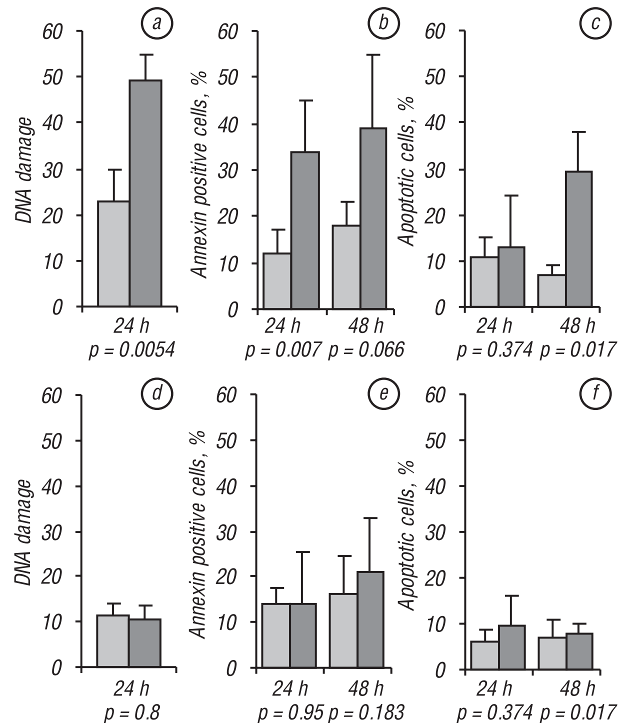

Fig. 2. Genotoxic and cytotoxic effects of MNU on PHA stimulated (*a, b, c*) and resting (*d, e, f*) human blood lymphocytes. Light-grey columns — intact lymphocytes, dark-grey columns — MNU/O6-bzG (500/20 µM) treated lymphocytes. *a* and *d*: DNA damage (double strand breaks); *b* and *e*: frequencies of apoptotic cells detected by annexin V-FITC complex at 24 and 48 h after MNU treatment; *c* and *f*: the same as *b* and *e* but apoptosis was measured according to morphological criteria

Genotoxicity of MNU on lymphocytes from patients with gynecological cancer. Although number of studied persons does not permit to compare strictly distributions for the case and control persons, mean values of DNA damage reveal significant difference between them. Fig. 3 shows that average number of DSBs in peripheral blood lymphocytes derived from patients with gynecological cancers is about 4-fold lower than that for lymphocytes of healthy donors ($p = 0.044$) but significantly higher than for MMR-deficient colorectal tumor HCT116 cells. MMR-deficiency of HCT116 cells is a result of mutation in *hMLH1* that inactivates the protein encoded by this gene [20]. All cases studied here were unlikely to be familial cancers and appear not to harbour germline mutations in any MMR genes. Therefore the observed decrease of MMR activity in lymphocytes from cancer patients may be associated with decreased expression of MMR proteins due to abnormal methylation of MMR genes [21]. It should be pointed out that according to our data lymphocytes from cancer patients are characterized by decreased proliferative indeces compared to normal donors: 12.8 ± 11% *vs* 27.2 ± 8.1% ($p = 0.048$). As for sensitivity of proliferation to MNU, cancer patients were resistant to the mutagen treatment (proliferative indeces for intact *vs* treated lymphocytes were 12.8 ± 11% *vs* 12.4 ± 9.3%, $p = 0.93$) in contrast to normal donors (respectively, 27.2 ± 8.1% *vs* 18.7 ± 7.5%, $p = 0.06$). This result is in agreement with reduced MMR activity in case lymphocytes compared to control cells.

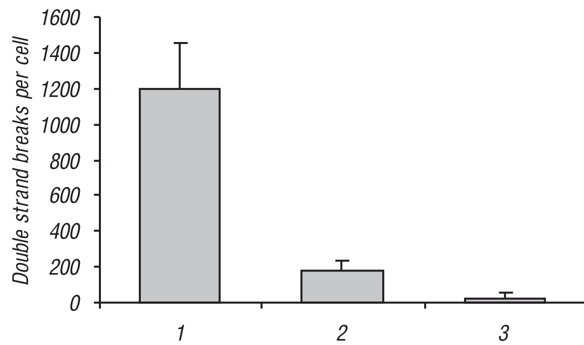

Fig. 3. Average numbers ± SEM of double strand breaks induced 24 h after MNU (500 µM) pulse-treatment of cells. 1 — PHA stimulated lymphocytes derived from health donors (n = 9), 2 — the same from patients with gynecological cancers (n = 10), 3 — MMR-deficient HCT116 cells (n = 6). 1 h before MNU treatment, O6-bzG (20 µM) was added to cells

If the observed reduction of functional activity of MMR in lymphocytes is not only associated with cancer, but is a preceding event, then genotoxic sensitivity of peripheral blood lymphocytes to MNU may serve as a marker and predictor for the risk of development of gynecological cancer [22].

ACKNOWLEGMENT

This work was supported by Russian Foundation for Basic Research, grant № 04-04-48453.

REFERENCES

1. **Spitz MR, Hsu TC.** Mutagen sensitivity as a marker of cancer risk. Cancer Detection Prevention 1994; **18**: 299–304.

2. **Berwick M, Vineis P.** Markers of DNA repair and susceptibility to cancer in humans: an epidemiologic review. J Natl Cancer Inst 2000; **92**: 874–97.

3. **Wu X, Gu J, Grossman HB, Amos CI, Etzel C, Huang M, Zhang Q, Millikan RE, Lerner S, Dinney CP, Spitz MR.** Bladder cancer predisposition: a multigenic approach to DNA-repair and cell-cycle–control genes. Am J Hum Genet 2006; **78**: 464–79.

4. **Schottenfeld D.** Epidemiology of endometrial neoplasia. J Cell Biochem Suppl. 1995; **23**: 151–9.

5. **Tronov VA, Kramarenko II, Karpukhin AV.** Colorectal cancer: mismatch repair eficiency, genomic instability, tolerance to apoptosis, assessment of cancer risk. Vopr Onkol 2005; **52**: 159–66.

6. **Vasen HFA, Watson P, Mecklin JP, Lynch HT.** New clinical criteria for HNPCC (Lynch syndrome) proposed by the International Collaborative Group on HNPCC. Gastroenterology 1999; **116**: 1453–6.

7. **Hickman MJ, Samson LD.** Role of DNA mismatch repair and p53 in signaling induction of apoptosis by alkylating agents. Proc Natl Acad Sci USA 1999; **96**: 10764–69.

8. **Kaina B, Ochs K, Grosch S, Fritz G, Lips J, Tomicic M, Dunkern T, Christmann M.** BER, MGMT and MMR in defense against alkylation-induced genotoxicity and apoptosis. Progr Nucl Acid Res Mol Biol 2001; **68**: 41–54.

9. **Tronov VA, Kramarenko II, Smirnova TD, Terekhov SM.** Comparision of geno- and cytotoxicity of methylnitrosourea on MMR-proficient and MMR-deficient human tumor cell lines. Cytology (Rus) 2006; **48**: 19–27.

10. **Tronov V, Konstantinov E, Petrakou E, Tsilimigaki S, Piperakis S.** Nicotinamide "protects'' resting lymphocytes exposed to hydrogen peroxide from necrosis but not from apoptosis. Cell Biol Toxicol 2002; **18**: 359–67.

11. **Konca K, Lankoff A, Banasik A, Lisowska H, Kuszewski T, Gozdz S, Koza Z, Wojcik A.** A cross-platform public domain PC image-analysis program for the comet assay. Mutat Res 2003; **534**: 15–20.

12. **Holley WR, Chatterjee A.** Clusters of DNA induced by ionizing radiation: formation of short DNA fragments. I. Theoretical modeling. Radiat Res 1996; **145**: 188–99.

13. **Kaina B, Christmann M.** DNA repair in resistance to alkytlating anticancer drugs. Int J Clin Pharm Ther 2002; **40**: 354–67.

14. **Mayer C, Popanda O, ZeleznyO, von Brevern M-C, Bach A, Bartsch H, Schmezer P.** DNA repair capacity after irradiation and expression profiles of DNA repair genes in resting and proliferating human peripheral blood lymphocytes. DNA Repair 2002; **1**: 237–50.

15. **Tronov VA, Konstantinov EM, Kramarenko II.** Role of excision mechanisms of DNA repair in induction of apoptosis. Biochemistry (Moscow) 2002. **67**: 730–6.

16. **Beranek DT.** Distribution of methyl and ethyl adducts following alkylation with monofunctional alkylating agents. Mutat Res 1990; **23**: 11–30.

17. **Kaina B, Fritz G, Conquerelle T.** Contribunion of O6-alkylguanine and N-alkylpurines to formation of sister chromatide exchanges, chromosomal aberrations, and gene mutations: new insights ganed from studies of genetically ingineered mammalian cell lines. Envir Mol Mutagen 1993; **22**: 283–92.

18. **Tatsuka M, Ibeanu GC, Izumi T, Narayan S, Rainana CV, Kim NK, Kang W, Roy G, Mitra S.** Structural organization of the mouse DNA repair gene, N-methylpurine-DNA glykosylase. DNA Cell Biol 1995; **14**: 37–45.

19. **Bignami M, O'Driscoll M, Aquilina G, Karran P.** Unmasking a killer: DNA *O*6-methylguanine and the cytotoxicity of methylating agents. Mutat Res 2000; **462**: 71–82.

20. **Parsons R, Li GM, Longley MJ, Fang WH, Papadopoulos N, Jen J, de la Chapelle A, Kinzler KW, Vogelstein B, Modrich P.** Hypermutability and mismatch repair deficiency in RER+ tumor cells. Cell 1993; **75**: 1227–36.

21. **Gifford G, Paul J, Vasey PA, Kaye SB, Brown R.** The acquisition of *hMLH1* methylation in plasma DNA after chemotherapy predicts poor survival for ovarian cancer patients. Clin Cancer Res 2004; **10**: 4420–6.

22. **McShane LM, Altman DG, Sauerbrei W, Taube SE, Gion M, Clark GM.** Reporting recommendations for tumor marker prognostic studies (Remarks). Exp Oncol 2006; **28**: 99–105.

ЧУВСТВИТЕЛЬНОСТЬ ЛИМФОЦИТОВ ЧЕЛОВЕКА К ГЕНОТОКСИЧЕСКОМУ ДЕЙСТВИЮ МЕТИЛНИТРОЗОМОЧЕВИНЫ: ВОЗМОЖНАЯ СВЯЗЬ С ОПУХОЛЯМИ ЖЕНСКОЙ ПОЛОВОЙ СФЕРЫ

Цель: **исследование эффекта метилнитрозомочевины (МНМ) на ФГА-стимулированные лимфоциты и в состоянии покоя здоровых доноров и пациенток с опухолями женской половой сферы.** ***Методы:*** **цитотоксический эффект МНМ (апоптотическая гибель лимфоцитов) оценивали по двум показателям — взаимодействию клеток с комплексом annexin V-FITC и по морфологическим изменениям ядра клеток после их окрашивания смесью 2 ДНК-тропных красителей. Генотоксический эффект МНМ, вторичные двунитевые разрывы ДНК (ДР) определяли с помощью метода нейтральных ДНК-комет. Использовали стандартный вариант метода и модифицированный для подсчета комет, формируемых только делящимися лимфоцитами, меченными BrUdr. Долю таких комет представляли как индекс пролиферации клеток.** ***Результаты:*** **лимфоциты в состоянии покоя устойчивы к гено- и цитотоксическому эффектам МНМ. В пролиферирующих клетках в ответ на воздействие МНМ формировались вторичные разрывы ДНК ($p < 0,01$) и возрастала частота появления апоптотических клеток ($p < 0,05$). Генотоксический эффект МНМ на стимулированные лимфоциты больных онкологического профиля в 4 раза ниже, чем на лимфоцитах здоровых доноров. Лимфоциты пациенток не изменяли пролиферативный индекс в ответ на действие МНМ; в лимфоцитах здоровых доноров действие МНМ вызывало 2-кратное снижение пролиферативного индекса.**
Выводы: **установлена связь между гено- и цитотоксическим ответом лимфоцитов на действие МНМ и наличием опухоли. Предполагается, что генотоксический эффект МНМ связан с процессом пострепликативной коррекционной репарации.**
Ключевые слова: **опухоли женской половой сферы, лимфоциты, коррекционная репарация (MMR), метилирующие агенты, двунитевые разрывы ДНК.**

Copyright © Experimental Oncology, 2006