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# Enhancement of Chromosome Aberrations in Lymphocytes of Mice after *In Vivo* Exposure to Chemicals and *In Vitro* Challenge with Bleomycin

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*ABSTRACT* : **Exposure to environmental toxicants can cause cellular problems including the interference of DNA repair processes which may lead to the development of cancer. The existence of toxicant-induced DNA repair abnormality was investigated using mice exposed *in vivo* to genotoxic chemicals and then challenging their exposed lymphocytes *in vitro* with bleomycin. The repair of bleomycin-induced DNA damage as estimated by the frequency of chromosome aberrations was determined. Our data indicates that the observed aberration frequencies after *in vivo* exposure to N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) and *in vitro* challenge with bleomycin are consistently higher than expected. The enhanced response is not due to the induction of chromosome damage by 25 or 50 mg/kg MNNG since the chemical did not cause chromosome aberrations in lymphocytes of these mice. The observed response after the combined exposure to benzo[a]pyrene (BP) and bleomycin was significantly lower than expected with low *in vivo* doses of BP (50 mg/kg) and then significantly higher than expected with the high doses (200 mg/kg). We interpret our data to indicate that *in vivo* exposure to genotoxic agents can cause abnormal DNA repair activities. The response is, however, independent of the clastogenic activities of the inducing chemicals, but dependent upon the inducing agents and on the exposure doses.**

**Key words : genetic toxicology, chromosome aberrations, abnormal DNA repair response, N-methyl-N'-nitro-N-nitrosoguanidine, benzo[a]pyrene, bleomycin.**

## INTRODUCTION

Excessive exposure to environmental toxicants causes a host of cellular problems which can lead to the development of long-term health problems such as cancer. Some of the consistently identified cellular problems are DNA damage, chromosome aberrations and gene mutation at the HPRT locus [Tice *et al.*, 1996]. However, expression of these biomarkers is not sufficient to claim the initiation of the carcinogenic process nor to provide precise prediction of cancer outcome. Additional biomarkers are needed to compliment the existing ones for a better understanding of health effects [Albertini *et al.*, 1996].

In our laboratory, we have been conducting population studies to detect the induction of chromosome aberrations from excessive exposure to environmental toxicants [Au *et al.*, 1991, Au *et al.*, 1995a, Au *et al.*, 1995b]. In addition, these studies were conducted to test our hypothesis that chronic exposure to toxicants can cause DNA repair problems [Au *et al.*, 1993a] leading to induction of the hypothesized "mutator phenotype" or genetic instability in carcinogenesis [Loeb *et al.*, 1994, Brentnall *et al.*, 1995]. We believe that repeated exposure to toxicants which causes the formation of DNA adducts in the genome can disrupt the DNA repair process and/or induce mutations in genes which are involved in the repair process. Since the DNA repair machinery is a complex system requiring numerous repair enzymes and their essential cofactors for the many repair pathways [Fadlallah *et al.*, 1994], the system represents an enormous target for damage from exposure to

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toxicants. In addition, damage to some of the common factors, e.g. ligase and topoisomerase, may cause repair problems in multiple pathways. We have tested our hypothesis by investigating the existence of abnormal DNA repair response in exposed populations using our challenge assay. In these studies, lymphocytes from donors were irradiated *in vitro* with gamma-rays and the frequency of chromosome aberrations was determined. Cells from individuals exposed to toxicants were expected to repair the radiation-induced DNA damage abnormally compared with those from unexposed controls. The repair abnormality is expected to cause increased chromosome aberrations in cells from toxicant-exposed populations in our challenge assay. In our experience, we found that lymphocytes from cigarette smokers, butadiene exposed workers and from residents living around uranium mining sites have significantly abnormal DNA repair response [Au *et al.*, 1991, Au *et al.*, 1995a, Au *et al.*, 1995b], Whereas, cells from mothers having neural tube defect babies have normal repair response [Au *et al.*, 1996]. In addition, the abnormal DNA repair response in butadiene exposed workers is confirmed as abnormal DNA repair activities in a study using a host cell reactivation assay in the same population [Hallberg *et al.*, 1996]. We interpret our findings to indicate that chronic exposure to environmental mutagens can cause abnormal DNA repair whereas exposure to teratogen does not.

In conducting population studies, we also observed variations in individual responses to toxicant exposure. This phenomenon is not unexpected because the human population is made up of heterogeneous individuals. In order to validate the existence of toxicant-induced DNA repair abnormality, we have initiated a study using our challenge assay in experimental animal. In this study, mice were treated with genotoxic chemicals acutely then lymphocytes were removed, cultured and treated with a DNA damaging agent, bleomycin (a direct-acting radiomimetic clastogen). The exposure sequence and protocol in mice are similar to those in our population studies with the exception of acute vs chronic exposures. Chronic exposure studies in mice will be conducted in the future. The chemicals chosen for *in vivo* treatments were N-methyl-N'-nitro-N-nitrosoguanidine (a direct-acting non-clastogenic mutagen) and benzo[a]pyrene (a clastogenic mutagen requiring metabolic activation). Chromosome aberrations in these cells were quantitated. Data from our study mdicates that the toxicant-induced enhanced response exists but the response depends on the type of inducing agents and on the exposure doses.

## Materials and Methods

### Chemicals

N-methyl-N'-nitro-N-nitrosoguanidine (MNNG, CAS No, 70-25-7) was purchased from Fluka Chemical Company, Swizerland. Benzo[a]pyrene (BP, CAS No.50-32-8) was obtained from Sigma Chemical Company, St.Louis, MO, USA and bleomycin was supplied by Dong-A Pharmaceutical Company, Seoul, Korea. MNNG and BP were dissolved in dimethyl sulfoxide and bleomycin was dissolved in distilled water. Fresh solutions were prepared for each experiment.

### Animals

Male C57BL/6 mice (15~20 gm) were obtained from the Animal Center of the Seoul National University (Seoul, Korea). Upon arrival, they were housed in our animal facility with free access to standard rodent chow and water and maintained in chambers with laminar air flow. The atmosphere in the chambers was maintained at a relative humidity of $55\pm7\%$ and a temperature of $22\pm1^{\circ}C$. Mice were kept in our facility for at least one week before they were used in our experiment.

### Treatment conditions

Mice were selected from our colony and assigned randomly to control and experimental groups. There were 5 mice per dose group. Control mice received solvent and experimental mice received a single dose of different concentrations of MNNG or BP i.p. in volumes of 0.1 ml/25 gm body weight. At 36 hr after the administration, mice were sacrificed by cervical dislocation. Spleens were removed aseptically and lymphocytes were released from spleens by squashing them with the blunt end of sterile syringes as we have done before [Au *et al.*, 1991, Heo *et al.*, 1995]. The cell counts from lymphocyte suspensions were determined and cell cultures were set up using standard conditions [Au *et al.*, 1991, Heo *et al.*, 1995]. At 24 hr after initiation of cultures, bleomycin was added to cultures to achieve a final concentration of 3 ug/ml. Eighteen hr later, mitotic cells were blocked with colcemid and cultures were harvested. Cytological preparations were made, stained and used to determine the frequency of chromosome aberrations.

One hundred metaphase cells per mouse were analyzed

under the microscope for documentation of chromosome aberrations. Since there were 5 mice per group, a total of 500 cells were therefore analyzed per experimental condition. The data were classified into % aberrant cells having any type of chromosome aberrations, chromatid breaks/100 metaphase cells and chromosome deletions/100 metaphase cells. From evaluation of the score sheets, the frequencies of chromatid exchanges and chromosome translocations were so low that they were not used for further evaluations.

### Statistical analysis of the data

The data for each experiment were entered in a general linear model parameterized using a cell mean model. To make comparisons among groups, linear contrasts of the estimated cell means were constructed. All p-values refer to the F-test generated from these linear constructs. All analyses were conducted using the SAS [The SAS system, 1992] statistical procedure REG.

## RESULTS

A summary of the chromosome data is shown in Table 1. As shown in the Table, exposure to MNNG *in vivo* did not induce any significant increase of % aberrant cells, chromatid breaks/100 cells and chromosome deletions/100 cells with any of the two treatment doses (25 and 50 mg/kg). Treatment with the challenging agent, bleomycin, *in vitro* caused a significant increase of chromosome aberrations in the three categories (from 0.8 to 10.4; 0.8 to 10.8 and 0 to 3.2, respectively; $p<0.05$). Data from cells having the combined *in vivo* and *in vitro* treatments indicate that the aberration frequencies from all combination groups had significant increase over the control group (for the 25 mg/kg MNNG dose, $p<0.0001$, 0.0001, 0.02, respectively; for 50 mg/kg dose, $p<0.0001$, 0.0001, 0.0001, respectively).

Treatment of mice with BP induced increases in % aberrant cells, chromatid breaks/ 100 cells and chromosome deletions/ 100 cells. Significant increase in % aberrant cells over the control was detected with the high treatment dose of 150 and 200 mg/kg ($p<0.03$ and 0.07, respectively) but not with the low doses of 50 and 100 mg/kg. In the combined (*in vivo/in vitro*) treatment groups, there was a BP dose-dependent increase in response and significant differences from the untreated controls for most of the experimental conditions (see Table 1).

Linear contrasts were constructed to compare the observed and expected frequencies of aberrations from the combined treatment groups. The expected frequencies were determined by adding the observed frequencies from each of the treatment groups assuming additive effects, For example, the expected frequency of % aberrant cells from the group treated with 25 mg/kg MNNG and 3 μg/ml

**Table 1.** Chromosome aberrations in mouse lymphocytes after *in vivo* exposure to chemicals and *in vitro* challenge with bleomycin[1]

| Chemical[2] | Bleomycin (μg/ml) | % Aberrant cells (S.D.) | Breaks/100 cells (S.D.) | Deletions/100 cell (S.D.) |
|---|---|---|---|---|
| MNNG (mg/kg) | | | | |
| 0 | 0 | 0.8 (1.1) | 0.8 (1.1) | 0 (0) |
| 0 | 3 | 10.4[3] (4.3) | 10.8[3] (5.0) | 3.2[3] (1.1) |
| 25 | 0 | 1.2 (1.1) | 0.8 (1.1) | 0.4 (0.9) |
| 25 | 3 | 17.2[3] (7.7) | 12.4[3] (6.4) | 3.2[3] (2.3) |
| 50 | 0 | 1.6 (1.7) | 1.2 (1.8) | 0.4 (0.9) |
| 50 | 3 | 21.2[3] (3.6) | 15.2[3] (4.6) | 5.6[3] (3.9) |
| BP (mg/kg) | | | | |
| 50 | 0 | 4.0 (2.0) | 2.4 (2.6) | 0 (0) |
| 50 | 3 | 7.7[3] (2.7) | 7.0 (5.2) | 2.0 (1.4) |
| 100 | 0 | 5.2 (1.8) | 2.4 (2.6) | 0.4 (0.9) |
| 100 | 3 | 15.2[3] (2.3) | 15.2[3] (5.8) | 2.8 (2.7) |
| 150 | 0 | 6.8[3] (3.6) | 2.8 (2.3) | 1.6 (1.7) |
| 150 | 3 | 15.3[3] (4.0) | 12.7[3] (5.0) | 4.7[3] (1.2) |
| 200 | 0 | 6.0[3] (4.0) | 5.6 (4.3) | 0.4 (0.9) |
| 200 | 3 | 22.4[3] (10.8) | 27.3[3] (16.4) | 10.3[3] (10.1) |

[1]5 mice were treated per experimental condition and 100 metaphase cells were analyzed per mouse.
[2]MNNG=N=methyl-N'-nitro-N-nitrosoguanidine; BP=benzo[a]pyrene.

**Table 2.** Observed compared with expected frequencies of chromosome aberrations after *in vivo* exposure to chemicals and *in vitro* challenge with bleomycin[1]

| Chemical | % Aberrant cells | | Breaks/100 cells | | Deletions/100 cells | |
|---|---|---|---|---|---|---|
| | Observed | Expected | Observed | Expected | Observed | Expected |
| MNNG (mg/kg)[2] | | | | | | |
| 25 | 17.2 | 11.6 | 12.4 | 11.6 | 3.2 | 3.6 |
| 50 | 21.2[3] | 12.0 | 15.2 | 12.0 | 5.6 | 3.6 |
| BP (mg/kg) | | | | | | |
| 50 | 7.4[3] | 14.4 | 7.0 | 13.2 | 2.0 | 3.2 |
| 100 | 15.2 | 15.6 | 15.2 | 13.2 | 2.8 | 3.6 |
| 150 | 15.3 | 17.2 | 12.7 | 13.6 | 4.7 | 4.8 |
| 200 | 22.4 | 16.4 | 27.3[3] | 16.4 | 10.3[3] | 3.6 |

[1]5 mice were treated per experimental condition and 100 metaphase cells were analyzed per mouse.
[2]MNNG=N-methyl-N'-nitro-N-introsoquanidine; BP=benzo[a]pyrene
[3]Significantly different from the expercted value (Student's t-test; $p<0.05$).

bleomycin is 1.2 (from the 25 mg/kg MNNG group)+l0.4 (from the group treated with 3 μg/ml bleomycin in vitro) to give 11.6. As indicated on Table 2, the observed frequency for the same group is 17.2. The linear contrast then [1.2+ 10.4−17.2]=−5.6 with p=0.08 which indicates no significant difference. For the two combined treatment groups with MNNG and bleomycin, the observed frequencies of abnormalities were consistently higher than the expected results, with the exception of the 25 mg/kg MNNG group in the deletions/100 cells category. For the 50 mg/kg MNNG group, the difference between the observed and the expected % aberrant cells is highly significant ($p<0.007$).

For the combined treatment group with BP and bleomycin, the observed frequencies for % aberrant cells, breaks/100 cells and deletions/100 cells were lower than the expected frequencies for the group treated with 50 and 100 mg/kg BP (Table 2). The observed frequency for the 50 mg/kg group is significantly lower than expected Student's t-test. The observed and expected frequencies were similar for the group treated with 150 mg/kg BP. For the group treated with 200 mg/kg BP, the observed frequencies for breaks/100 cells and deletions/100 cells were significantly higher than the expected frequencies ($p<0.03$).

## DISCUSSION

Our data suggests that *in vivo* exposure to an acute dose of genotoxic agents can cause abnormal response to an *in vitro* challenge from a clastogenic agent, bleomycin. In most cases, the response is towards enhancement of the challenge effects. For example, using MNNG (the non-clastogenic mutagen), the observed frequencies of chromosome abnormalities are consistently higher than the expected frequency. The response is not linked directly with the induction of chromosome aberrations from MNNG since none of the tested doses induced observable increase of chromosome aberrations. On the other hand, the observed frequencies from the combined exposure to BP and to bleomycin are lower than the expected ones at low BP exposure doses and then become significantly higher at the high BP dose. The data from the BP exposed group suggests the existence of adaptive response at low dqses and synergistic response at the high doses. Again, the response is not linked directly to the induction of chromosome aberrations by BP because all tested doses of BP consistently caused higher chromosome aberrations than the control. The data therefore indicates that *in vivo* exposed cells express genotoxic responses to an *in vitro* challenge in specific manners: the responses are independent of the clastogenic activities of the *in vivo* inducing chemicals but are dependent on the types of genotoxic agents and on the *in vivo* exposure doses.

There are several possible explanations to the ephanced response as observed. One possibility is the persistence of DNA adducts which interferes with DNA repair processes after the *in vitro* challenge. Simple alkylation adducts such as those induced by MNNG are mostly removed very quickly leaving minor adducts which are not well recognized by cellular repair mechanisms or which are located in difficult to reach regions of DNA [Fadlallah *et al.*, 1994, Souliotis *et al.*, 1995]. We believe that these adducts interfere with DNA repair processes leading to unrepaired DNA strand breaks (chromosome breaks) and mistakes in rejoining broken DNA strands (chromosome

translocations) [Au *et al.*, 1993b, Au *et al.*, 1996b].

BP forms bulky DNA adducts and their removals are not as quickly as those formed by MNNG [Au *et al.*, 1996b, Suk *et al.*, 1995]. On this bases, it is therefore possible that at low doses of BP, repair of the easily removable adducts was still going on in the lymphocytes at the time of the *in vitro* challenge with bleomycin. These "primed" cells were therefore able to repair the DNA damage caused by bleomycin leading to the phenomenon of adaptive response. The phenomenon has been documented in human lymphocytes which were irradiated with very low doses of radiation followed by a much higher challenge dose [Olivieri *et al.*, 1984]. With high doses of BP, it is possible that the high number of DNA adducts overwhelm the DNA repair mechanisms leading to the higher than expected responses.

Another possibility is the mutagenic activities of the inducing agents. Both MNNG and BP are mutagenic [Tice *et al.*, 1996, Rasouli *et al.*, 1994]. Treatment of cells *in vitro* with MNNG was reported to cause the induction of chromosome aberrations in the second cell cycle after the treatment [Gallaway *et al.*, 1995]. These authors proposed that the phenomenon was due to misincorporation of nucleotides during the initial repair in the first cell cycle followed by mistakes in repairing the mismatched base pairs during the second cell cycle. We feel that damage to the DNA repair machinery is possible because it is an enormous target. In addition, the repair process involves multiple enzymes, many co-factors and some overlapping pathways, therefore damage to only a few of the required genes will affect the fidelity of the repair process [Friedberg *et al.*, 1995]. We also believe that it is not necessary to damage identical genes in a large number of cells to cause a detectable abnormal repair response. It is possible that damage to a number of required genes, especially whose enzymes are needed in multiple pathways (e.g. ligase), each in different cells will cause a detectable overall abnormal response.

As indicated earlier, adaptive responses have been observed in some individuals [Olivieri *et al.*, 1984], however, the phenomenon is not consistently documented [Wojcik *et al.*, 1995a]. The variable response may be due to the heterogeneous make up of the human population with different life-style and exposure to environmental toxicants. The heterogenous adaptive response is documented in studies using a small number of monkeys which were caught in the wild [Guedeney *et al.*, 1989]. In their *in vivo-in vitro* radiation exposure experiments, these authors observed enhanced chromosomal radiosensitivity initially, then the phenomenon of reduced dicentric frequencies in 6/22 cases and altered acentric frequencies in 16/22 cases. A lack of adaptive response is reported in mouse splenocytes as measured by the lack of enhanced unscheduled DNA synthesis [Wojcik *et al.*, 1995b]. The lack of enhanced repair activities is consistent with our documentation of increased chromosome aberrations in our study of *in vivo* exposure and *in vitro* challenge. Therefore, our data indicates that *in vivo* exposure to toxicants can cause worsened responses to subsequent exposure to another genotoxic agent. The observation has important implications to public health and environmental toxicology.

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# MNNG 또는 Benzo(a)pyrene 유도 염색체이상에 미치는 Bleomycin의 효과

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환경독성물질에 의한 폭로는 세포내 **DNA**의 수복과정에 영향을 미쳐 돌연변이나 암을 유발할 수 있다. 독성물질에 의해 유도된 **DNA**의 비정상 수복효과를 판단하기 위하여 ***in vivo***에서 **MNNG** 또는 **Benzo(a)pyrene**을 투여하고 ***in vitro***에서 **Bleomycin**을 처리하여 나타나는 염색체이상효과를 관찰하였다. 실험결과, **MNNG**를 투여 후 **Bleomycin**을 처리하였을 때 염색체이상의 상승효과가 나타났다. 한편, **Benzo(a)pyrene** 투여 후 **Bleomycin**을 처리하였을 때는 높은 농도에서 염색체이상의 상승효과가 나타났다. 이같은 결과는 **MNNG**나 **Benzo(a)pyrene** 같은 유전독성물질들이 ***in vivo***에서 세포내 비정상 **DNA** 수복을 일으킬 수 있으며, 이러한 작용은 관련 유전독성물질의 염색체손상성에 무관하며 투여용량에 의존되는 것으로 판단된다.