EUR. J. ONCOL.; Vol. 19, n. 1, pp. 5-12, 2014

The BLM-3AB challenge assay: a micronuclei-based method for measuring DNA Repair Capacity

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Summary. Efficient DNA repair activity is critically important in maintaining genomic integrity and reducing the risk of cancer. Hence, having sensitive DNA repair functional assays can be useful for identifying such abnormality and can be applied to risk prediction and disease prevention. We have developed a version of the challenge assay to measure DNA repair capacity. The assay is based on the cytochalasin B-micronucleus assay, with DNA damage induced by bleomycin and the subsequent DNA repair mechanism inhibited by 3-aminobenzamide. We used the assay to measure the DNA repair capacity of five vinyl chloride-exposed workers, five benzene-exposed workers and five healthy controls. We found that the DNA repair capacity in both vinyl chloride and benzene-exposed populations were higher than the controls, the mean index being 0.12, 0.16 and 0.54, respectively. For each individual, the micronuclei (MN) frequency of the baseline, being treated with bleomycin only and bleomycin + 3-aminobenzamide (BLM-3AB) together increased gradually. These data support the feasibility of the assay. In conclusion, our assay provides a new tool to identify DNA repair capacity of different individuals and may be applied in pathogenic mechanism-related research, especially occupational-related carcinogenesis.

Key words: challenge assay, DNA repair, mutagen sensitivity, micronuclei, population monitoring

«Saggio di prova BLM-3AB: un metodo basato sui micronuclei per misurare la capacità riparativa del DNA»

Riassunto. L'efficienza dei sistemi di riparazione del DNA è cruciale per il mantenimento dell'integrità genomica e nel ridurre il rischio di cancro. Quindi, avere a disposizione test sensibili per la valutazione funzionale dei sistemi di riparazione del DNA può essere utile per identificare le anomalie e tali test possono essere applicati nella previsione del rischio e nella prevenzione delle malattie. Abbiamo sviluppato una versione di prova del saggio per misurare la capacità di riparazione del DNA. Lo studio è basato sul saggio citocalasina B-micronuclei, con danno al DNA indotto da bleomicina e conseguente meccanismo di riparazione inibito da 3-aminobenzamide. Abbiamo utilizzato il saggio per misurare la capacità di riparazione del DNA in 5 lavoratori esposti a cloruro di vinile, in 5 lavoratori esposti a benzene ed in 5 lavoratori non esposti ed in salute come controlli. Abbiamo riscontrato che la capacità di riparazione del DNA sia nella popolazione esposta a cloruro di vinile che a benzene è stata più alta rispetto ai controlli, l'indice medio è stato rispettivamente di 0,12, 0,16 e 0,54. Per ogni individuo, la frequenza dei micronuclei (MN) di base viene incrementata in modo graduale sia attraverso il trattamento con sola bleomicina che con bleomicina + 3 aminobenzamide insieme. Questi dati hanno supportato l'attuabilità del saggio. In conclusione, il nostro saggio fornisce un nuovo strumento per identificare la capacità di riparazione del DNA di differenti individui e può essere applicato nella ricerca correlata a meccanismi patogenici, specialmente nella cancerogenesi occupazionale.

Parole chiave: saggio di prova, riparazione del DNA, sensibilità mutagenica, micronuclei, monitoraggio della popolazione

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Introduction

Human beings are continuously exposed to chemical and physical agents that can cause DNA damage and cancer. Fortunately, cells have developed a series of defense systems that facilitate repair of inflicted damage but the defense mechanism is not fool-proof. This point is well-illustrated by populations with inherited DNA repair abnormality (1, 2), by the multiple mutations in carcinogenesis (3, 4) and by chronic exposure to environmental mutagens (5, 6).

As a measure of DNA repair capacity (DRC), the challenge assay which was developed by our co-author, Au (7) has been widely used around the world. The assay is based on irradiating lymphocytes *in vitro* from toxicant-exposed and control populations, and determining the subsequent expression of chromosome aberrations. The assay has been used to indicate DRC among exposed populations and their risk of developing cancer.

In the traditional challenge assay, cell samples (usually peripheral leukocytes) from cancer patients or subjects exposed to an agent (for in vivo studies), or cells cultured in the presence of a specific genotoxic agent (for in vitro studies), are induced to develop lesions in their DNA by some agent of known potency. Subsequently, the DNA damage is assessed at different times using various genotoxicity tests. The most usual procedure is to measure at time 0 (immediately after treatment with genotoxic agent) and after a repair time. Then the decrease in genotoxic damage evaluated after the repair time with regard to the damage evaluated prior to repair time is taken as a measure of DRC. However, for some tests, such as the cytochalasin B-micronucleus assay (CBMN) assay we adopt in our protocol, we are not able to detect the right damage level immediately after induction of genotoxic damage, so we propose a surrogate method.

In our assay, we propose the following: we use bleomycin (BLM) as the challenge agent to induce DNA damage. In addition, if repair activity is inhibited right after BLM treatment, the damage will remain unrepaired, with the other parallel culture experiencing the normal repair process, so that the relatively high frequency of MN after repair inhibition will be due to the inefficiency of cells in repairing the damage caused. We have named the new method the BLM-3AB assay. This means that the decreased MN frequency in the control cells can be attributed to the normal repair system of the individual. The stronger the DNA repair capacity, the bigger the different MN frequencies between cell cultures from the same individual with and without the repair inhibitor treatment. Additionally, we can calculate a proportional value to quantify the relative DNA repair capacity of each individual. Thus, a big value means a high DNA repair capacity.

The selection of a proper repair inhibitor is critical for the development of the assay. 3-Aminobenzamide (3AB) is a well studied inhibitor of poly (ADP-ribose) polymerase (PARP), an enzyme implicated in the maintenance of genomic integrity (8). Inhibition of PARP enzyme by 3AB under nontoxic conditions (low concentrations and short period of exposure) in combination with genotoxic agents leads to phenotypic transformation (9). Moreover, it has been demonstrated that PARP depletion increases the genomic instability of cells exposed to genotoxic agents (10). These observations provide sound support for the possible application of 3AB in our protocol.

In this report, we describe the development and validation of a new functional DNA repair assay.

Materials and methods

Briefly, cell samples (usually peripheral leukocytes) from cases and controls were exposed *in vitro* to BLM, a well-studied S-independent radiomimetic agent that induces double-strand breaks in DNA (11). Subsequently, the cell culture was divided into two tubes, one having 3-AB added to suppress the DNA repair responses and the other allowed to perform normal repair function. The DNA repair capacity was then estimated using the cytochalasin B-micronucleus (CBMN) assay.

1 Materials

1.1 Laboratory supplies

(1) Sterile 5-ml Vacutainer™ CPT™ tubes containing sodium heparin as the anticoagulating agent (green top tubes) for the collection of peripheral blood from donors.

Standard supplies include: needles and syringes for blood collection, 15 ml sterile conical centrifuge tubes, sterile pipettes, 500 ml sterile flasks, microscope slides with frosting at one end for writing labels, coplin jars, hand counter, sterile and non-sterile gloves, etc.

1.2 Equipment

(1) Laminar air flow cabinets for handling sterile procedures such as blood culture and BLM and 3AB treatment.
(2) Binocular research light microscopes that have a minimum of 100× and 1000× magnification.
(3) Centrifuge.
(4) CO_2 incubator (REVCO HABITAT™).

1.3 Reagents

(1) Bleomycin (BLM, Sigma Chemical Co.).
(2) Cytochalasins B (Cyt-B, Sigma Chemical Co.).
(3) 3-Aminobenzamide (3-AB, Sigma Chemical Co.).
(4) Other chemicals include: potassium chloride, methanol, glacial acetic acid.
Gurr Giemsa stain (Sigma Chemical Co.)
Phosphate buffer solution.
Sterile dual steaming water.

1.4 Cell culture

The cell culture components were 4 ml of culture medium (RMPI 1640) supplemented with 1 ml 10% fetal calf serum, 1% penicillin (100 U/ml) - streptomycin (100 μg/ml) and 3% phytohemagglutinin (PHA).

1.5 Reagent setup

1.5.1 Cytochalasin (Cyt-B):
(1) Stock solution: 25 mg in 12.5 ml dimethyl sulfoxide (DMSO) to give a Cyt-B solution concentration of 2000 μg/ml as stock solution. The solution was stored at -20°C for up to 12 months.
(2) Final solution: just before the addition of Cyt-B, the stock solution was diluted with sterile normal saline to give a final Cyt-B solution concentration of 300 μg/ml.

1.5.2 Bleomycin (BLM)
(1) Stock solution: 1 mg solid was dissolved in 0.5 ml sterile normal saline to give a BLM solution concentration of 2000 μg/ml, then the solution was stored at -20°C.
(2) Final solution: Just before BLM treatment, the stock solution was diluted with sterile normal saline to give a final concentration of 151.5 μg/ml (added 12.2 ml sterile normal saline per ml stock solution).

1.5.3 3-Aminobenzamide (3AB)
(1) 250 mg solid was dissolved in 36.72 ml sterile normal saline to give a 3AB solution concentration of 50 mmol/l, then stored at -20°C.
(2) The solution was mixed gently and during the following operation, 5ml cell culture medium was added with 125 μl to make an ultimate ml of 1.25 mmol/l.

2 Method

2.1 Recruitment of study subjects

Depending on the objectives of our investigation, certain genotoxicants such as vinyl chloride and benzene exposed populations and healthy controls were recruited as study subjects. It was of great importance to collect detailed information from each individual carefully by questionnaires or other means. The contents of questionnaires covered personal information (age, gender, etc.), life styles (smoking habits and alcohol consumption), health history, exposure history and family history. It must be noted that individuals exposed to factors that may affect the effect biomarker, such as intake of therapeutic drugs, should be excluded in advance. All the individuals had to sign their informed consent for ethical reasons.

2.2 Blood sampling

Fresh blood samples were collected from the study subjects by venipuncture into 5 ml tubes with

heparin as anticoagulant. The tubes were then shaken to mix the heparin with the blood. The blood tube for each volunteer was labeled with a single code number and maintained in an ice box. The published evidence available suggested that storage of blood between 5 and 22°C for up to 24h has no significant impact on baseline or radiation-induced MN frequency (12), These tubes were transported to the processing laboratory in less than 6 hours.

2.3 Dosage determination

We conducted repeated trials to establish the optimal doses for the use of BLM and 3-AB. The dose selection was based on the evaluation of best cell activity and moderate cell toxicity.

We collected blood samples from three volunteers (coded as A, B and C, respectively) in our laboratory and treated them with different doses of regents for dose selection. First, we explored the proper ultimate concentration of BLM. The toxic effect of different concentrations is listed in Table 1.

According to the above results, we chose 1 μg/ml as the final concentration of BLM in our protocol, and went on to determine the concentration of 3-AB, the trial data being shown in Table 2.

From the data we drew the conclusion that the repair of DNA damage which was induced by 1.0 μg/ml BLM was moderately inhibited by 3-AB at a concentration of 1.25 mmol/l. Our trials resulted in an ultimate concentration of 1 μg/ml and 1.25 mmol/l for BLM and 3-AB, respectively.

Table 1. Toxic effect of different concentration of BLM treatment among three normal adult volunteers

Treatment concentration		MN frequencies (‰)		
BLM (μg/ml)	3-AB (mmol/l)	A	B	C
0	0	0	1	3
0.4	0	1	1	3
0.8	0	4	3	6
1.0	0	6	7	10
1.2	0	15	13	-
1.5	0	-	-	-

- With few cells observed

Table 2. Inhibition effect of different concentration of 3-AB treatment among three normal adult volunteers

Treatment concentration		MN frequencies (‰)		
BLM (μg/ml)	3-AB (mmol/l)	A	B	C
1.0	0	6	7	10
1.0	0.50	6	7	11
1.0	1.00	8	8	13
1.0	1.25	10	13	15
1.0	1.50	-	12	-

- With few cells observed

2.4 Cell culture

Using sterile procedures throughout with all operations in Laminar Air Flow cabinets.

(1) 1 ml anti-coagulated blood was added to a tube which contained the complete cell culture medium. The medium contained 4 ml of RMPI 1640 medium which was supplemented with 1 ml 10% fetal calf serum, penicillin (100 U/ml), streptomycin (100 mg/ml) and 3% PHA.
(2) The cultures were then placed in a CO_2 incubator, which was set at 37°C with a humidified atmosphere and with 5% CO_2.
(3) 24 hours later, culture samples were treated with BLM to an ultimate concentration of 1 mg/ml.
(4) After further incubation at 37°C for 30 minutes, the cultures were washed twice in 5 ml of medium (RMPI 1640) in a centrifuge at 800 r/min for 10 min to terminate the effect of BLM.
(5) At the end of the pulse treatment with BLM, the cells were split into two test tubes, each with 5 ml of culture medium, one of which having 3-AB added at 1.25 mmol/l. The other parallel culture was used as a control.
(6) The two cultures from the same donor were then placed in the incubator for another 20 hours.
(7) 100 ml Cyt-B final solution (with an ultimate concentration of 300 mg/ml, dissolved by DMSO and diluted with sterile normal saline) was added to each 5 ml cell culture to give a

final concentration of 6 mg/ml. This step was performed 44 hours after PHA stimulation of lymphocytes.

(8) Cultures were returned to the incubator and incubated until harvesting.

2.5 Harvesting of cells using centrifugation

24–28 h after addition of Cyt-B, the cells were harvested for slide preparation and scoring according to the procedure given below. The harvest time chosen was designed to maximize the proportion of binucleate (BN) cells and minimize the frequency of mononucleated and multinucleated (three or more nuclei) cells.

(1) The cultures were transferred into 5 ml tubes and then gently resuspended. The cultures were then spun in a centrifuge at 800 r/min for 10 min.
(2) The supernatant medium was discarded.
(3) The packed cell layer received 3 ml of pre-warmed (37°C) 0.075M KCl hypotonic solution. The cells were resuspended immediately and then incubated for 10~15 min, 37°C for hypotonic treatment.
(4) 4~5 ml ice-cold fixative (3:1 ratio of methanol/glacial acetic acid) was added at the top of tubes after hypotonic treatment, and mixed immediately. As a result, the cell suspension turned black from red, indicating lysis of red blood cells.
(5) The tubes were centrifuged at 800~1000 r/min for 10 minutes.
(6) The supernatant was discarded.
(7) 5 ml of fixative at 4°C was added to the cell button. The cells were immediately resuspended was and then incubated at room temperature or on the ice for 15~20 min.
(8) The tubes was centrifuged at 800~1000r/min for another 10 minutes and the supernatant was discarded.
(9) Steps 7 and 8 were repeated. By this time the cell pellet would be clean and white and they would be ready to make slides.

2. 6 Slide preparation

The slides were washed and flushed with double distilled pure water in advance and dipped inside the ice water for use.

(1) The supernatant in the tube fixative was removed with about 0.5~1 ml left. The cell suspensions were used to drop onto slides.
(2) A drop of cell suspension was dispensed onto the surface of the wet slides from a height of about 15~20 cm. The cell suspension in fixative then reacted vigorously with the water-based solution and spread evenly over the slides. The slides with cells were placed on clean paper towers to absorb the excess fluid and facilitate drying. If possible, a well-ventilated hood was highly preferred for conducting the operation.
(3) The slides were placed vertically on clean trays for drying in an airy and non-dusty environment for one night.
(4) The air-dried slides were stained with 5% Gurr Giemsa stain (5ml Gurr Giemsa stain diluted with 95 ml sterile water and filtered through Whatman Filter paper.
(5) Stained slides were examined to assess the efficiency of staining and the cell density. To calculate the MN frequency, it was necessary to have at least 1000 BN cells to score.
(6) If the staining and the density of the cells were satisfactory, the slides were left to dry completely for at least 30 min before putting on coverslips.

2.7 Coverslipping and storage

(1) The slides were placed on tissue or paper to be coverslipped and one coverslip was set alongside each.
(2) One or two large drops of DePex were placed on each of the coverslips in the area corresponding to the cell spots.
(3) The slides were inverted over the coverslip and thus the DePex was allowed to spread by capillary action. The coverslip was slid gently to and fro to expel any excess DePex and air bubbles. We had to ensure that the spots did not have air bubbles over them.

(4) The excess DePex was wiped from the edges of the slide.
(5) The slides were then placed on a tray and left overnight in the fume hood to dry.
(6) The slides were finally stored in slide boxes at room temperature for microscopic analysis.

2.8 Examination of slides and assessment of DRC

(1) Micronucleus were examined at 400× magnification using a light microscope. The number of micronuclei (MNi) in 1000 (BN) lymphocyte cells needed to be scored and the frequency of MNi per 1000 BN cells calculated. The criteria for scoring MNi were according to the standard described by Fenech (13, 14).
(2) After counting the different MN frequency of the cell culture from each individual before and after 3-AB treatment, the DRC (3AB index) was represented with a relative proportional value calculated by the following equation:
$3AB\ index=(MN_{-BLM+3AB}-MN_{-BLM})/\ MN_{-BLM+3AB}$
where $MN_{-BLM+3AB}$ was the MN frequency after applying 3AB, while MN_{-BLM} meant the MN frequency of the counterpart control.

3. Measurement of DNA repair capacity

3.1 Sample recruitment

The assay was conducted on three groups of population: five VC-exposed workers employed in a VC polymerization plant in Shanghai, China; five benzene-exposed workers from a shoe factory and five school teachers without any history of exposure to any known toxicants.

3.2 Results

We measured the DRC of individuals from the three groups using the BLM-3AB challenge assay mentioned above. The results are described in Table 3. For each individual, the MN frequency increased from baseline, treated with BLM only and BLM+3AB together. The overall measured DRC varied in the three sub-populations with different environmental conditions. The mean 3AB index for VC exposures, BZ

Table 3. Characteristics of study subjects and DRC

Group	No.	Age(year)	Gender	Smoking	Drinking	MN (‰)	MN_{-BLM} (‰)	$MN_{-BLM+3AB}$ (‰)	3-AB index
VC exposures	1	52	F	NS	ND	12	25	26	0.04
	2	50	M	S	D	14	24	26	0.08
	3	37	M	NS	D	8	25	28	0.11
	4	37	F	NS	ND	3	12	15	0.20
	5	42	M	S	D	4	10	12	0.17
BZ exposures	6	38	F	NS	ND	9	13	15	0.13
	7	49	M	S	D	4	10	11	0.09
	8	50	F	NS	ND	4	6	7	0.14
	9	42	M	S	D	4	16	22	0.27
	10	38	M	S	D	5	15	18	0.17
Controls	11	57	M	S	ND	1	4	8	0.50
	12	53	F	NS	ND	1	3	6	0.50
	13	57	M	S	D	2	5	9	0.44
	14	47	M	NS	D	2	5	11	0.55
	15	34	F	NS	ND	0	3	7	0.57

Note: Gender: M-male F-female; Smoking: S-smoker NS-non-smoker; Drinking: D-drinking; ND-non-drinking; MN: MN frequency without either BLM or 3-AB treatment; MN-BLM: MN frequency with BLM treatment;
MN-BLM+3AB: MN frequency with BLM treatment + 3-AB treatment

exposures and controls were 0.12, 0.16 and 0.54, respectively.

Conclusions

In summary, the main purpose of this paper was to introduce a version of the challenge assay based on micronuclei, and apply it in different populations to test the reliability and practicality of the new method.

One major advantage of our method is its adoption of the relatively convenient and reliable CBMN assay to detect genetic damage. The traditional mutagen sensitivity assay was followed by the Comet assay. The CBMN assay allows excellent precision because the data obtained are not confounded by altered cell division kinetics caused by cytotoxicity of agents tested or sub-optimal cell culture conditions. The method is now applied to various cell types for genetic damage monitoring, as well as screening of chemicals for genotoxic potential (11).

As an established and widely accepted methodology, the reproducibility of the assay can be ensured. Besides, all the slides were coded so that the observer was unaware of the status of the subject and scoring was done "blind". In order to avoid inter-observer differences, all the samples were analyzed by one investigator, when the scores were completed, and all the images were re-analyzed jointly by both investigators in order to obtain a consensus. Thus, all the MNi were analyzed following the same criteria. So the 3AB index calculated by our method is credible.

It cannot be denied that our study sample was relative small and we were not able to conduct statistical analysis, though we only applied the method to VC and BZ exposure. Whether this assay can also assess response to other types of carcinogens needs to be evaluated in other prospective large-scale epidemiological population studies. To this end, we are now extending our study to other occupationally exposed populations with a high risk of developing tumors.

Therefore, though it needs to be validated by further studies, this simple reproducible and rapid as well as cost-effective assay also provides a new tool for identifying DNA repair capacity. The DNA repair capacity of an organism may be both an effect indicator and a susceptibility indicator, and is therefore a step toward our objective of identifying subgroups with low DNA repair capacity and elevated cancer risk so as to protect the health of workers. Additionally, the assay can be used on different types of volunteers for other purposes, such as in certain types of cancer patient to identify certain susceptible subpopulations.

Acknowledgements

The study was supported by the Natural Science Foundation of China (30671740, 81072280), 973 Program (2011CB503802) and Shanghai Bureau of Public Health (08GWD12, 08GWZX0402). We thank physicians Shang-jian Chai and Jun Li for their help with physical examination of the workers and data collection of vinyl chloride exposure.

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Received: 2.10.2013
Accepted: 13.1.2014
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