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# Rejoining kinetics of bleomycin-induced DNA single-strand breaks in agarose-bound human blood cells

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ABSTRACT

The rejoining kinetics of individual DNA single-strand breaks (SSBs) are difficult to measure, in biomonitoring studies, because SSB rejoining is rapid and hard to control. We have detected early (0, 1, 2, 5, 10 and 30 min) events in SSB rejoining in human leukocytes, with the alkaline comet assay, at a low concentration of bleomycin (BLM; 0.5 μg/ml). Background Tail DNA% (percentage of DNA that remained in the comet tail) of the subjects was 1.23% (25th–75th percentile: 0.72–1.64). BLM treatment increased this to 62.4% (25th–75th percentile: 57.8–70.4) at $t = 0$, decreasing to the background level by 30 min. Analysis of 45 subjects showed that the fastest return to the background level occurred in 5 min, whereas the slowest return took approximately 30 min. The early rejoining kinetics of SSBs may show multiple patterns, varying among individuals.



## 1. Introduction

Since the introduction of the alkaline single-cell gel electrophoresis (or alkaline comet) assay in 1988 [1], scientists have used this method for assessment of DNA damage and repair in genotoxicology [2–4] and biomonitoring studies [5–7]. However, a basic problem remains: precise control of the rejoining of induced DNA single-strand breaks (SSBs). Singh et al. [1] noticed this problem and attempted to solve it by exposing agarose-embedded lymphocytes on microslides to X-ray or $H_2O_2$ before cell lysis. However, these researchers did not explain how one might control the rejoining of DNA SSBs. In the following years, some studies have applied a similar method, but failed to observe early kinetics of DNA SSB rejoining [8–12]. To date, most researchers treat samples one-by-one in culture dishes that contain the test substance (possibly allowing DNA repair in fresh medium at 37 °C), collect cells by centrifugation, stop exposure to the test substance by washing, and adjust the cell numbers before sandwiching the cells in a microgel (37 °C∼42 °C) for the alkaline comet assay [13]. Because the manipulation of cells before cell lysis usually requires at least 30 min, rejoining of DNA SSBs could occur, and the measured results represent what damage remains after such rejoining [14]. To observe a significant influence of the test substance, researchers are forced to use higher concentrations of test substances.

This problem may be important in biomonitoring studies, because the concentrations of substances in the environment are usually low. When blood samples are used, the isolation of nucleated blood cells would be laborious [15] and likely to allow the isolated cells to rejoin their DNA SSBs in a buffer environment. In some cases, the isolation itself may induce unpredictable DNA damage [16]. Thus, some scientists suggest the use of whole blood rather than isolated lymphocytes in biomonitoring studies [17].

In the present study, we directly sandwiched fresh whole blood cells obtained from 45 male Japanese adults in agarose, before BLM treatment; processed the agarose-bound blood samples in batch; and observed DNA rejoining (mainly of SSBs) kinetics in the leukocytes by use of the alkaline comet assay.

## 2. Subjects and methods

### 2.1. Subjects

All subjects signed an informed consent form, and the study was approved by the Ethics Committee of Osaka University Medical School. The subjects were 45 Japanese male factory workers at a hard-metal industry in Osaka. 44 individuals were clerks or technicians and one was a manufacturing worker. Average age was 43.8 ± 10.9y.

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**Table 1**
Alteration in TailDNA% during different rejoining periods after BLM treatment of agarose-bound peripheral blood leukocytes from 45 subjects.[a]

| TailDNA% | Rejoining Period | | | | |
|---|---|---|---|---|---|
| | 0~1 min (%) | 1~2 min (%) | 2~5 min (%) | 5~10 min (%) | 10~30 min (%) |
| Decreased | 30 (66.7) | 25 (55.6) | 43 (95.6) | 26 (57.8) | 43 (95.6) |
| Unchanged | 4 (8.9) | 5 (11.1) | 2 (4.4) | 3 (6.7) | 2 (4.4) |
| Increased | 11 (24.4) | 15 (33.3) | 0 (0) | 16 (35.6) | 0 (0) |
| Total | 45 (100) | 45 (100) | 45 (100) | 45 (100) | 45 (100) |

[a] The decreases, lack of changes and increases in the TailDNA% of a subject were judged by comparison with the adjacent median of the TailDNA% of 80~120 leukocytes using the Kolmogorov–Smirnov test at $p \leq 0.05$.

### 2.2. Alkaline comet assay, in batch mode

Blood samples were collected in the morning and sandwiched in agarose on microslides, using a previously described method [18]. In brief, we collected blood (5 mL) from fasting workers (20~63 years of age) during their annual health check-up via a peripheral vein, using coded heparinized vacuum tubes (VENOJECT®II VP-H050 K, Terumo, Tokyo, Japan) between 8:30 a.m. and 10:30 a.m. Blood samples were maintained in an ice-box until preparation of the microslides between 2:00 and 6:00 p.m. on the same day. Before binding the blood cells to agarose on 76 × 26-mm frosted glass microslides (Matsunami, Osaka, Japan), we precoated the frosted sides with 0.75% normal-melting agarose, 20 μL, (Sigma) in PBS (pH 7.2, $Ca^{++}$- and $Mg^{++}$-free) and dried the microslides. Before loading the blood samples, we spread 0.75% normal-melting agarose, 80 μL, with a 50 × 24-mm cover glass. After solidification at 4 °C for 10 min, we sealed the glass microslides with a piece of an 8-well CoverWell™ perfusion chamber (Funakoshi, Tokyo, Japan). For each sample, we gently mixed whole blood, 2.5 μL, with 0.75% low-melting agarose (Cambrex Bio Science, Rockland, USA), 147.5 μL, in PBS (pH 7.2, $Ca^{++}$- and $Mg^{++}$-free) and sandwiched the cell suspension layer (10 μL) between the preloaded agarose and another layer of 0.75% low-melting agarose (theoretically 500~1500 cell/well). We loaded four samples, each in duplicate, on each microslide. After gently removing the piece of CoverWell™ perfusion chamber, we arranged the microslides in a rack that could hold 20 microslide pieces. We prepared seven batches of microslides; each batch had 45 samples. For the untreated batch (negative control, background), we directly put the microslides into lysis buffer at 4 °C. For the BLM-treated batch (positive control, repair time zero), we immersed the microslides in ice-cold (4 °C) RPMI 1640 (Sigma) solution containing 0.5 μg/mL bleomycin (BLM) within 1 s, treated them for 5 min, removed them and dipped them twice into ice-cold $ddH_20$ and moved them to lysis buffer at 4 °C within 5 s. For the repair batches, we removed the BLM-treated microslides, dipped them twice into ice-cold RPMI 1640, and moved them to RPMI 1640 at 37 °C within 5 s. The microslides were maintained in RPMI 1640 at 37 °C for exactly 1, 2, 5, 10, and 30 min. The microslides were then removed, immediately dipped twice into ice-cold $ddH_2O$, and moved to lysis buffer at 4 °C within 5 s. The microslides were then denatured, electrophoresed and neutralized as previously described [18]. After staining with 10 μg/mL ethidium bromide for 1 min, we quantified the DNA damage of 40~60 leukocytes from each well under a microscope at 100×magnification using a Comet Assay IV imaging system (Perceptive Instruments, Haverhill, UK) attached to a fluorescent microscope (Olympus, Tokyo, Japan) and used the "Tail%DNA" (tail intensity) to present the nuclear DNA damage for each leukocyte (Fig. 1).

### 2.3. Data

For each subject at each observation time point (background and after 0, 1, 2, 5, 10, and 30 min of DNA SSB rejoining), we obtained approximately 80~120 leukocyte "Tail%DNA" data points and used

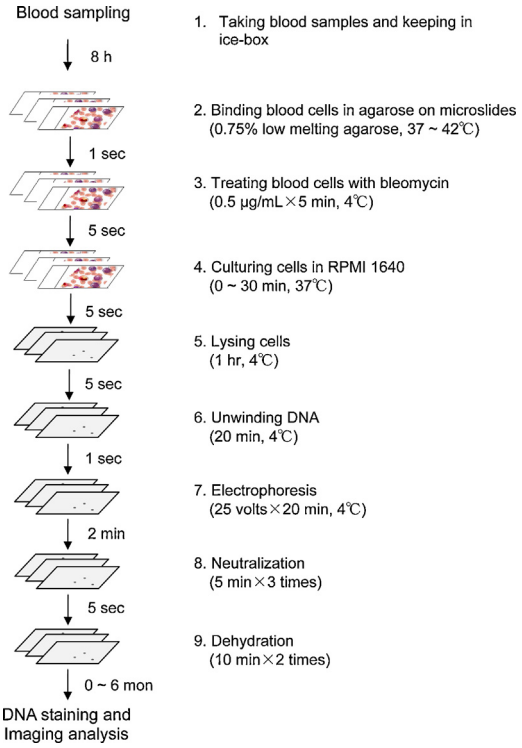


**Fig. 1.** Scheme for detecting bleomycin (BLM)-induced DNA damage and rejoining kinetics in human whole blood bound to agarose on microslides. The number next to the down-facing arrow is the time interval between adjacent steps. BG: background; LMA: low-melting agarose.

the median (25th–75th percentile) of the data to represent the individual DNA damage level (defined as individual "TailDNA%"). For all of the subjects at each observation time point, we obtained 45 individual "Tail%DNA" data points and used the median (25th–75th percentile) to represent the total DNA damage level of the 45 subjects (defined as group "TailDNA%"). In the study, we collected a total of 37,403 "Tail%DNA" data points to obtain 315 individual "TailDNA%" and seven total "TailDNA%" data points for analysis.

### 2.4. Statistics

All of the data were analyzed using Stata12 (StataCorp LP, College Station, TX, USA). The level of statistical significance between the medians was set at $p \leq 0.05$ in the Kolmogorov–Smirnov test (*K-S* test). All statistical tests were two-sided.

## 3. Results

The seven total "TailDNA%" results represent the DNA damage levels of the subjects at each observation time point (background and after 0, 1, 2, 5, 10, and 30 min of DNA SSB rejoining). Background "TailDNA%" was 1.23% (25th–75th per-