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An optimized method for detecting gamma-H2AX in blood cells reveals a significant interindividual variation in the gamma-H2AX response among humans

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ABSTRACT

Phosphorylation of histone H2AX on serine 139 (gamma-H2AX, γH2AX) occurs at sites flanking DNA double-strand breaks (DSBs) and can provide a measure of the number of DSBs within a cell. Here we describe a rapid and simple flow-cytometry-based method, optimized to measure gamma-H2AX in non-fixed peripheral blood cells. No DSB induced signal was observed in H2AX−/− cells indicating that our FACS method specifically recognized gamma-H2AX accumulation. The gamma-H2AX assay was capable of detecting DNA damage at levels 100-fold below the detection limit of the alkaline comet assay. The gamma-H2AX signal was quantitative with a linear increase of the gamma-H2AX signal over two orders of magnitude. We found that all nucleated blood cell types examined, including the short-lived neutrophils induce gamma-H2AX in response to DSBs. Interindividual difference in the gamma-H2AX signal in response to ionizing radiation and the DSB-inducing drug calicheamicin was almost 2-fold in blood cells from patients, indicating that the amount of gamma-H2AX produced in response to a given dose of radiation varies significantly in the human population. This simple method could be used to monitor response to radiation or DNA-damaging drugs.

INTRODUCTION

Ionizing radiation (IR) and many chemotherapeutic agents kill cancer cells by induction of DNA double-strand breaks (DSBs) (1). Since both strands are damaged, DSBs are more difficult to repair compared to base damage or single-strand DNA breaks (SSBs) that leaves one template strand intact to guide repair. Therefore, DSBs activate a distinct signaling system that is capable of detecting a few, possibly a single DSB (2,3). Activation of the DSB signal often results in cell death or permanent growth arrest. Since DSBs are more toxic in fast growing cells, agents that induce DSBs are often used to treat cancer. Most DSB inducers also generate large numbers of SSBs, which likely participate little to the toxic effect (1). In the case of IR, the level of SSBs is 20–40 times higher compared to the levels of DSBs. The agent that produces the highest relative levels of DSBs is the enediyne calicheamicin γ1 (CLM). This drug contains two radical centers that become positioned close to the opposing strands when it binds the minor groove of double-stranded DNA. Activation of the radical centers is cooperative, resulting in efficient cutting of both DNA strands (4). We have earlier shown that at least 30% of the CLM-induced strand breaks are DSBs in cellular DNA (5).

The clinical effects of DSB inducing agents are highly variable among individuals (6). Therefore, the same dose of IR can result in severe side effects in some cancer patients while other patients show no obvious sign of the delivered dose. It would therefore be advantageous if we could measure the DNA damage response in each patient and use this signal to guide the dosing. Previous attempts to measure DNA damage in patients undergoing chemotherapy have relied on the alkaline comet assay. In this assay, individual cells are molded into agarose on microscopic slides and exposed to an electrical field after alkaline in-gel lysis. The electrical field forces cellular DNA containing strand breaks to migrate from the nucleus generating a 'comet tail' that is proportional to the level of SSBs and DSBs in the cell (7,8).

An early event after introduction of DSBs is the phosphorylation of a special form of histone 2A, denoted H2AX that is part of 10% of all nucleosomes in the cell (9–11). Histone 2AX contains a distinct C-terminal extension, with a consensus phosphorylation

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at serine 139. The related DNA-activated kinases ATR, ATM and DNA-PK are responsible for the formation of several thousands of phosphorylated H2AX (γH2AX, H2AXS139ph (12) or gamma-H2AX) in a 2-Mbp region of the DSB, within minutes after its formation (10,13–17). Gamma-H2AX is specifically bound by MDC1 that assist in the assembly of several proteins, including ATM, the Mre11/Rad50/Nibrin complex and 53BP1 that subsequently participates in the spreading of gamma-H2AX formation in a 2-Mbp region from the DSB (18–20). This amplification of the locally generated DSB signal later assist in the globalization of the DSB response through massive ATM-mediated Chk2 activation (21) (reviewed in (22)). In yeast, the H2AX homolog H2A, is also phosphorylated in a DSB-dependent manner (23), and participates in the assembly of chromatin-remodeling complexes, called SwrC, Ino80 and NuA4. These complexes are required for the modulation of the chromatin structure that is observed surrounding a DSB and for normal resistance to IR (24,25). It is likely that the Tip60 complex has a similar chromatin modulating function in human cells (26). Mouse cells lacking H2AX show defects in the DSB response and DSB repair, which is especially pronounced at low levels of DSBs (27). This, and other lines of evidence, indicates that formation of gamma-H2AX is required for the proper amplification of the DSB response after low doses of radiation.

The local formation of gamma-H2AX allows microscopical detection of distinct foci by fluorescent gamma-H2AX-specific antibodies that most likely represent a single DSB (28–30). The potential to detect a single focus within the nucleus makes this the most sensitive method currently available for detecting DSBs in cells. This method is, however, labor-intensive and will be difficult to adapt in clinical practice. In contrast, flow cytometry allows simple detection of gamma-H2AX in a large number of cells (31). Several reports show that the level of gamma-H2AX as detected by flow cytometry correlates well with the number of DNA strand breaks, to the level of cell death and radiosensitivity (32–34).

We have developed a flow-cytometry-based method optimized for measurement of gamma-H2AX in non-fixed cells. Our assay was apparently specific for gamma-H2AX, since no DNA damage induced signal was obtained in H2AX−/− cells. The gamma-H2AX signal was linearly correlated with the level of DNA damage and detected hundred times lower levels of CLM-induced DNA damage compared to the alkaline comet assay. We find that all nucleated blood cells are able to induce gamma-H2AX, but to different levels. There was a significant interindividual difference in the gamma-H2AX signal at a given DNA damage level. This flow cytometry method can be used to measure the level of DSBs in patient cells, for example, in patients undergoing chemotherapy.

MATERIALS AND METHODS

Drug exposure and preparation of blood cells

Calicheamicin γ1 (CLM) was a generous gift from George Ellestad (Wyeth-Ayers Research), dissolved at 2 mM in DMSO in small fractions (Sigma), and stored at −70°C. Under these conditions, CLM showed no loss of activity. Repeated freeze and thaw cycles, especially at low concentration, was shown to inactivate CLM, and therefore avoided. The H2AX−/− and H2AX+/+ cells were a generous gift from Andre Nussenzweig (27). Blood samples, not older than 3 h, were collected from routine hematology laboratory, after routine full blood count analysis on a Celldyn cell counter (Abbott). Drugs were added directly to the blood, and the blood samples were incubated at 37°C under continuous rocking. For incubation with CLM, it was found that the maximum level of gamma-H2AX signal was reached after a 30-min of incubation. After incubation with drugs, the blood samples were kept on ice during preparation. Total white blood cells were prepared by mixing 70 µl of blood with 1.6 ml of ice-cold lysis solution (0.154 M NH_4Cl, 0.01 M $KHCO_3$, 0.09 mM EDTA, pH 7.3) on ice for 20 min. After centrifugation at 4000 rpm, 4 min at 0°C, the cell pellet was resuspended in 1 ml of ice-cold lysis solution for 10–20 min, and centrifuged as above. The cell pellet was suspended in 25 µl of PBS (1.9 mM NaH_2PO_4, 8.1 mM Na_2HPO_4, 154 mM NaCl, pH 7.2), supplemented with 1 g/l bovine serum albumin (BSA), (fraction V, Sigma) and stored on ice no longer than 3 h before analysis of gamma-H2AX, or by the comet assay. Lymphocytes were prepared by carefully pipetting 2 ml blood mixed 1:1 in PBS supplemented with 5 mM EDTA on a layer with 1.5 ml lymphoprep (AXIS-SHIELD PoC AS) in a 5 ml flow-cytometry tube. Centrifugation at 1200 g was for 10 min at 0°C. The lymphocyte layer was mixed with 1.3 ml of lysis solution, incubated for 20 min on ice and centrifuged as described for total white blood cell preparation (above). Neutrophils were prepared from the erythrocyte pellet obtained in the lymphoprep by removing the lymphoprep solution and resuspending the erythrocyte pellet 1:25 in lysis solution. After incubation on ice for 20–40 min the solution was centrifuged 1200 g for 10 min and the pellet resuspended in 1.5 ml lysis solution, incubated for 20 min and centrifuged as described for preparation of total white blood cells. Purified lymphocytes were frozen by resuspending the cells in PBS-Pi (PBS supplemented with 10 mM NaF, 1 mM Na_2MoO_4, 1 mM $NaVO_3$) at 1 million cells/ml and frozen in 10 µl fractions at −80°C. Upon analysis, 200 µl of Block8 was added directly to the frozen cells and processed as described below. Manual differential count of 200 cells after May–Grünwald stain showed that the lymphocyte preparation contained 94% lymphocytes, 6% monocytes and <0.5% neutrophils. The neutrophil preparation contained 98.5% neutrophils, 1.5% lymphocytes and <0.5% monocytes. Fixation with ethanol was done as described (31). Fixation with paraformaldehyde (PFA, Sigma) was done by suspending 100 000 WBC in 0.5 ml PBS, supplemented with 1% PFA, on ice for 10 min. Cells were collected by centrifugation, and washed twice with 1 ml PBS 1 g/l BSA. Blood (2.8 ml) in 5.2 cm petri dishes were irradiated on ice, with a Philips RT 100 X-ray machine, using an acceleration voltage of 100 kV and 13 Gy/min.

Flow cytometry measurement for gamma-H2AX

Staining for gamma-H2AX was done by adding 5000–100 000 cells to 150–200 µl of Block 8 (PBS supplemented with 1 g/l BSA, 8% mouse serum (Sigma), 0.1 g/l RNase A type XII-A (Sigma), phosphatase inhibitors (10 mM NaF, 1 mM Na_2MoO_4, 1 mM $NaVO_3$), 0.25 g/l sonicated herring-sperm DNA type XIV (Sigma), 0.1% Triton X100, 0.44 µg/l monoclonal anti-gamma-H2AX, FITC conjugate (Upstate biotechnology, 16-202A), 0.02% NaN_3). Incubation was for 4–20 h on ice in the dark, before dilution with 350 µl PFA dilution buffer (PBS supplemented with 2% PFA). The samples were analyzed directly on a FACSScan (Becton & Dickinson), with calibrations done using FITC-labeled plastic beads (Calibrite, Becton & Dickinson). Fluorescence intensity in arbitrary units was plotted in histograms, and the mean fluorescence intensity was calculated using Weasel version 2.3 software. To check for doublets, white blood cells (WBC) were prepared from several patients, and stained with propidium iodide in Block 8 and analyzed by flow cytometry. These tests consistently showed that doublet nuclei were <0.1%, and that no S- or G_2-phase cells were present.

Flow-cytometry-mediated sorting

WBC were prepared as described above after treatment with various concentration of CLM and 25 million cells were stained with anti-CD3 conjugated to activated peridinin-chlorophyll-protein (PerCP), and anti-CD14 conjugated to phycoerythrin. In a separate tube, 25 millon cells from each CLM treatment were stained with anti-CD20 conjugated to phycoerythrin (PE), and anti-CD23 conjugated to allophycocyanin (APC). All antibodies were from Becton and Dickinson. The labeled cells were sorted on a FACSAria (Becton and Dickinson), using settings suggested by the manufacturer. Neutrophils were sorted based on light scatter characteristics, intermediate CD14 expression. Monocytes were separated based on light scatter characteristics and strong CD14 expression. T-lymphocytes were selected based on light scatter characteristics and high CD3 expression. B-lymphocytes were selected based on light scatter characteristics, strong CD20 and CD23 expression and absence of CD3 expression. Sorted cells were collected in PBS, 1 g/l BSA. The entire sorting procedure was done at +4°C in 1 h. After sorting, the cells were concentrated by centrifugation and prepared for gamma-H2AX analysis and comet assay.

Comet assay

The comet assay was performed as described previously, with some modifications. Briefly, 5000–10 000 cells were mixed with 150 µl 0.75% low-melting agarose (Sigma type VII, USA), kept at 37°C. The agarose-cell suspension was spread onto pre-chilled gel bond film (Cambrex Bio Science Rockland). The preparations were left on a chilled plate for 5 min before lysis (0.03 M NaOH, 1 M NaCl, 2 mM EDTA, 0.5% *N*-lauryl sarcosine) for 1 h, and thereafter equilibrated (0.03 M NaOH, 2 mM EDTA) for 1 h. Electrophoresis of the agarose embedded cells was run at 0.67 V/cm for 15 min in the same solution. The film was then neutralized in 0.4 M Tris-HCl, pH 7.5, fixed in 99.5% ethanol for 2 h, and allowed to dry, usually overnight. After staining of the film with SybrGold (molecular probes) for 5 min, it was rinsed briefly in de-ionized water. Analysis of the DNA that migrated from the nuclei was done by visual scoring (35) or measurement of the percentage of total DNA in the comet tail by Comet III software (Perceptive Instruments Ltd).

RESULTS

Development of an optimized protocol for gamma-H2AX staining in white blood cells

EDTA-blood was treated *in vitro* with CLM to induce DSBs. Total white blood cells (WBC) or lymphocytes were prepared and subjected to different fixation protocols, and stained for gamma-H2AX using a FITC-labeled antibody. The FITC signal was measured in isolated cells using flow cytometry. It was found that the gamma-H2AX signal only increased 2–3-fold in CLM-treated WBC using published protocols (31). Gamma-H2AX is a nucleosomal protein, and therefore retained in the nucleus along with other histones when cells are lysed at physiological salt concentration. It was therefore reasoned that non-fixed nuclei could be stained with the FITC-labeled gamma-H2AX antibody. We tested this by mixing non-fixed, CLM-treated WBC with the FITC-labeled gamma-H2AX antibody in a detergent-containing buffer. Using this protocol we observed a 10-fold increase of the gamma-H2AX signal in CLM-treated WBC (Figure 1A). This showed that non-fixed cells were efficiently stained with the FITC-labeled gamma-H2AX antibody. In addition, no gamma-H2AX could be detected in extracts from CLM-treated cells under these conditions, indicating that no gamma-H2AX was lost from the nuclei during the staining procedure (data not shown). We conclude that it is possible to stain non-fixed cells for gamma-H2AX and that this procedure allows improved detection of gamma-H2AX in WBC.

Initially, the procedure included washing of the nuclei after the antibody staining, by centrifugation. This resulted in a high proportion of doublet nuclei, and large aggregates that prevented reproducible quantification of the FITC signal. Aggregation was avoided when washing of the nuclei was omitted, but resulted in a slightly increased background signal from WBC not treated with CLM.

To further optimize the gamma-H2AX-method, we undertook a survey of different blocking agents and staining procedures (Table 1). It was found that addition of sonicated chromosomal DNA, with a medium size of 100–300 bp, resulted in a reduction of the background signal, as well as an increase of the signal in CLM-treated cells (Figure 1B). The signal enhancement was less pronounced using similar amounts of a double-stranded 32-bp oligonucleotide (data not shown). We also found that high molecular weight DNA induced aggregation of nuclei, resulting in non-reproducible flow cytometry

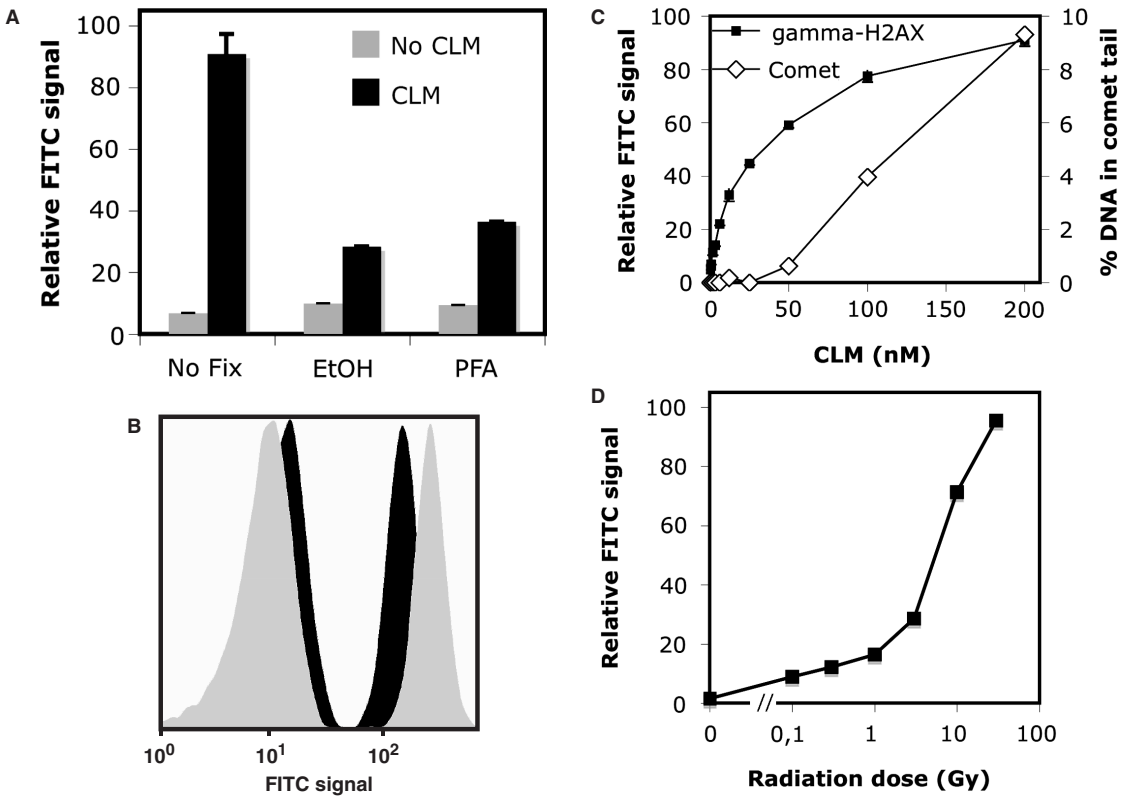

Figure 1. Optimization of gamma-H2AX measurement in WBC by flow cytometry. (**A**) Staining for gamma-H2AX does not require fixation. WBC was prepared from blood, not treated (No CLM), or treated with 160 nM CLM (CLM), and not fixed (No Fix), fixed with ethanol (EtOH), or fixed with PFA before staining for gamma-H2AX with a FITC-labeled gamma-H2AX antibody. The mean FITC intensity was measured by flow cytometry. (**B**) DNA in the blocking solution enhances the gamma-H2AX signal. Flow cytometry plot of WBC prepared from blood not treated (left), or treated with 160 nM of CLM (right). The cells were stained for gamma-H2AX in Block 8 without (black), or with (gray) of 0.25 mg/ml sonicated chromosomal DNA, and the FITC signal was measured by flow cytometry. (**C**) The gamma-H2AX signal increases linearly with DNA damage. Lymphocytes were prepared from blood samples treated with 0.5, 1.5, 3, 6, 12, 25, 50, 100 or 200 nM CLM for 30 min, and analyzed for gamma-H2AX and by the comet assay. Error bars represent variation among duplicate samples. (**D**) Blood pooled from 4 donors was irradiated with gamma radiation on ice before incubation at 37°C for 30 min. Lymphocytes were prepared and the resulting gamma-H2AX signal determined.

Table 1. Different ingredients effect on gamma-H2AX staining

Ingredient	Fold change in gamma-H2AX-signal
Phosphatase inhibitors	2.01
DNA 0.25 g/l	1.87
8% Mouse serum	1.71
16% Mouse serum	1.01
gamma-H2AX ab 0.4 mg/l	0.95
gamma-H2AX ab 0.3 mg/l	0.65
gamma-H2AX ab 0.2 mg/l	0.50
Okadaic acid 1 mg/l	1.05
Protease inhibitors	0.92
MgCl2 5 Mm	0.70
PFA fixation	0.30
EtOH fixation	0.21
DNaseI 0.2 g/l + MgCl2 5 mM	0.25
0.03% Triton X100	0.15
polylysine 0.1 g/l	0.07
PEG 6000 6%	0.02

Protease inhibitors were 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine-HCl, 1 μg/ml leupeptin, 1 μg/ml pepstatin A. Fold change in gamma-H2AX was calculated by dividing the mean FITC signal obtained in WBC, prepared from blood treated with 160 nM CLM, and stained in block 8, with or without the indicated alteration. For example, phosphatase inhibitors and DNA resulted in a 2-fold increase in the gamma-H2AX signal, whereas PFA fixation resulted in a 3-fold decrease of the gamma-H2AX signal.

results. Using the optimized blocking buffer (Block 8), the FITC signal increased with longer staining times up to 3 h, but prolonged staining up to 20 h did not result in further enhancement of the signal. Storage of CLM-treated blood on ice for 30 h before gamma-H2AX staining resulted in less than 15% reduction of the gamma-H2AX signal indicating that it is possible to store blood samples for one day without loss of the gamma-H2AX signal. CLM-treated WBC could be stored at −80°C in PBS for extended periods of time without loss of the gamma-H2AX signal. In contrast, if CLM-treated blood samples were frozen using several different protocols, WBC or lymphocytes retrieved from the frozen sample consistently lost the gamma-H2AX signal. Therefore, if the blood samples were not analyzed directly the sample could be stored on ice for 24 h, or lymphocytes prepared and stored at −80°C. The gamma-H2AX signal increased linearly with CLM concentrations from 0.5 to 10 nM, but started to level out at 50 nM (Figure 1C). The same cells were analyzed with the alkaline comet assay (Figure 1C). The result indicated that the gamma-H2AX method was able to detect 100-fold lower levels of CLM-induced DNA damage compared to the alkaline comet assay.

Lymphocytes prepared from blood exposed to different doses of ionizing radiation showed that the gamma-H2AX signal was detectable at doses as low as 0,1 Gy (Figure 1D). The gamma-H2AX assay was reproducible, and double samples varied typically with a CV below 5%. The Block 8 solution was stable for at least 2 months at +4°C. We conclude that measurement of the gamma-H2AX signal in non-fixed cells by FACS analysis is a convenient, sensitive and reproducible way to measure the DSB response in patient cells.

The FITC signal in response to DSBs specifically measures gamma-H2AX

To examine if the FITC signal specifically reflected accumulation of gamma-H2AX, mouse embryonic fibroblasts (MEFs) with (H2AX+/+) or without (H2AX−/−) H2AX expression (27) were treated in growth medium with various concentrations of CLM to induce DSBs. We observed that cleavage of cellular DNA by CLM was almost 10 times more efficient in growth medium compared to when CLM was added to blood (data not shown), possibly because CLM binds or is partly inactivated by some blood component. Therefore the gamma-H2AX signal was more pronounced at low CLM concentrations in this experiment compared to when CLM was added directly to blood (compare Figure 1C and Figure 2). In the absence of CLM, the gamma-H2AX signal was the same in both H2AX−/− and H2AX+/+ cells and not significantly higher compared to undamaged lymphocytes prepared from blood. As expected, H2AX+/+ MEFs showed a CLM-dose-dependent increase of the FITC signal. In contrast, the H2AX−/− MEFs failed to show any FITC signal over background even at doses of CLM that produced maximal FITC signal in H2AX+/+ cells (Figure 2). We therefore conclude that our flow-cytometry protocol specifically measures gamma-H2AX accumulation.

The gamma-H2AX signal in different blood cells

Next, we wanted to examine the gamma-H2AX signal in different blood cells. To this end, blood from a healthy subject was treated with different concentrations of CLM *in vitro*. WBC was prepared, and neutrophils, monocytes, T-lymphocytes and B-lymphocytes were separated by flow cytometry sorting. The cells were then stained for gamma-H2AX (Figure 3) or analyzed with the comet assay, to measure independently the levels of CLM-induced DNA strand breaks. The gamma-H2AX signal was present in all nucleated cell types examined. The gamma-H2AX signal and the comet signal were lower in neutrophils compared to monocytes and T-lymphocytes. The gamma-H2AX signal in the unsorted WBC showed an intermediate response, compared to neutrophils and lymphocytes, reflecting the fact that neutrophils constituted approximately 50% of the WBC population in this blood sample. B-lymphocytes showed 3 times higher level of gamma-H2AX signal, despite having similar amount of DNA damage as measured by the alkaline comet assay. However, B-lymphocytes constituted less than 5% of the WBC (data not shown) and therefore contributed little

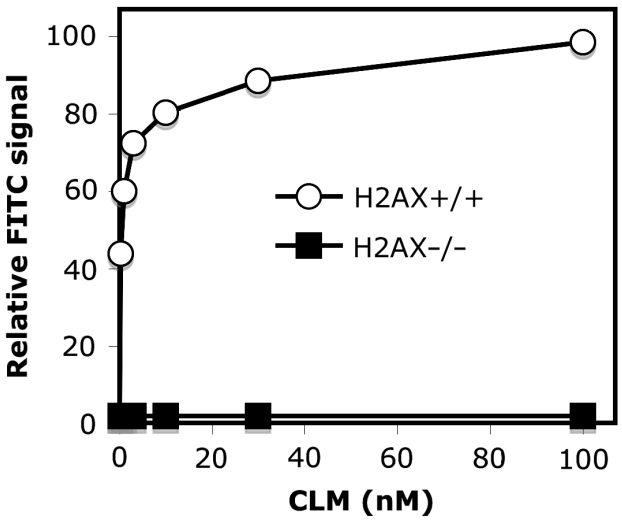

Figure 2. The gamma-H2AX assay specifically detects CLM-induced H2AX phosphorylation. Non-transformed mouse embryonic fibroblasts (MEFs) expressing H2AX (H2AX+/+) and MEFs lacking H2AX expression (H2AX−/−) were treated with different CLM concentrations in growth medium for 30 min before analysis with the gamma-H2AX assay.

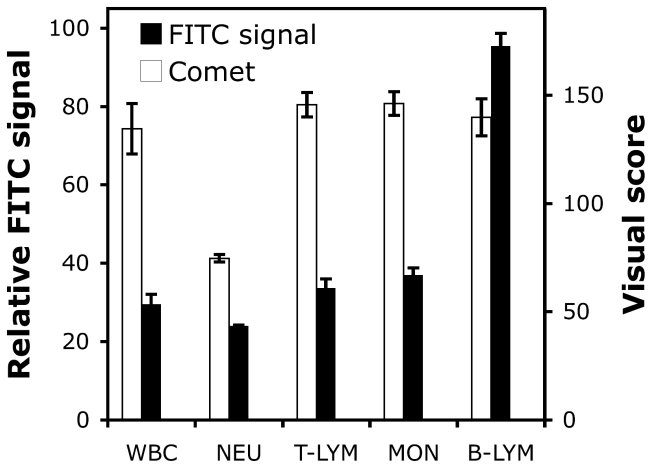

Figure 3. Gamma-H2AX signal in different blood cell populations. WBC was prepared from blood treated with 40 nM CLM, or 200 nM CLM. The cells were stained with different surface markers, and neutrophils (NEU), T-lymphocytes (LYM-T), monocytes (MON) and B-lymphocytes (LYM-B) were purified by flow cytometry sorting. Sorted cells treated with 40 nM CLM were stained for gamma-H2AX (black bars). Sorted cells from the 200 nM CLM treatment were subjected to the comet assay, to measure the levels of total DNA strand breaks. The comet assay results were analyzed using visual scoring. Error bars represent variation among duplicate samples.

to the signal from unsorted WBC. Therefore, the majority of unsorted WBC shows a homogenous gamma-H2AX induction. It is therefore possible to measure the DSB response in unsorted WBC cells.

Interindividual variation of the gamma-H2AX signal in response to DSB inducing agents

Next, we wanted to know if our gamma-H2AX assay could be used to measure the gamma-H2AX signal in

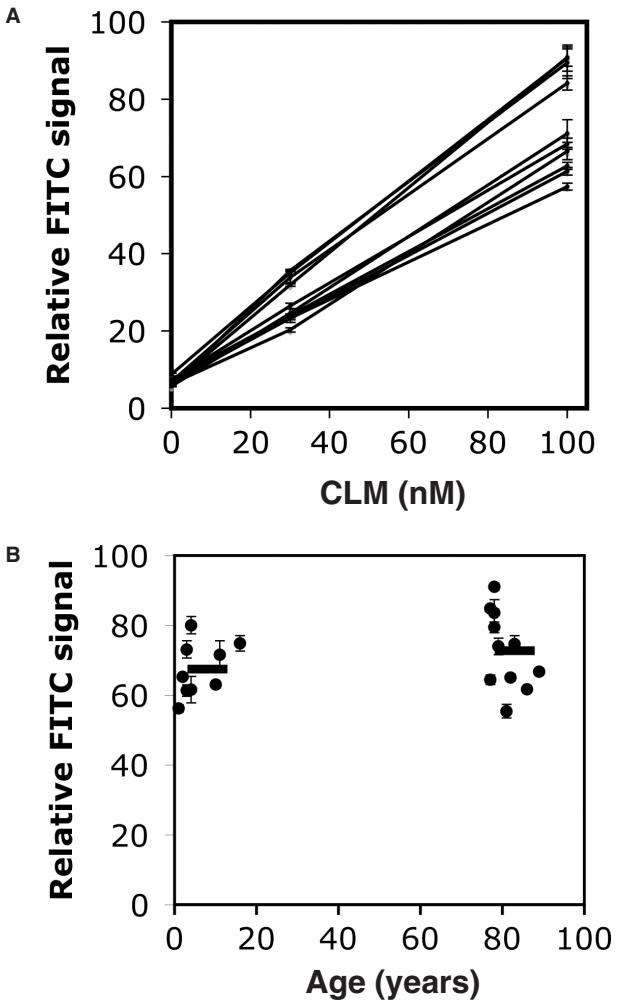

Figure 4. The CLM-induced gamma-H2AX signal in WBC shows significant variation among individuals. (**A**) Measurement of the gamma-H2AX signal in ten patients. Blood samples from ten patients were treated in duplicate with 0, 30 or 100 nM CLM. WBC were prepared, and the resulting gamma-H2AX signal was measured by flow cytometry. (**B**) No correlation between age and the gamma-H2AX signal. Blood samples from nine patients, with the age between 1 and 16 years, from a pediatric primary health care center, and blood samples from eleven patients 77–89 years old, admitted to the hospital, were treated in duplicate with 40 nM CLM. WBC were prepared, and the gamma-H2AX signal measured. A horizontal line indicates the mean gamma-H2AX signal in each age group. Error bars represent the difference in mean FITC signal intensity between double samples.

patient samples. To this end, we first collected blood from ten patients admitted to the hospital for medical reasons unrelated to cancer, and treated this blood *in vitro* with two different concentrations of CLM. WBC cells were prepared and stained for gamma-H2AX (Figure 4A), or prepared for DNA strand break measurements, using the comet assay (Table 2). The results showed that the gamma-H2AX signal varied almost 2-fold among the highest and the lowest values, with a CV of 18%. There was no correlation between the level of strand breaks, as measured by the comet assay and the gamma-H2AX signal ($R^2 = 0.2$). In addition, we failed to find any

Table 2. Analysis of the gamma-H2AX signal in response to CLM in ten patients

Patient	Age	Sex	gH2AX at 30 nM	gH2AX at 100 nM	Comet at 500 nM CLM (visual score)	WBC (10^9/l)	NEU%	LYM%	MON%	TPK (10^9/l)	HGB (g/l)
1	88	M	78 +/− 1	200 +/− 7	263	7.12	48.5	35.6	12.5	268	97.3
2	56	F	52 +/− 1	127 +/− 2	246	3.23	46.9	38.9	13.3	106	121
3	82	M	52 +/− 2	136 +/− 2	260	12.9	66.6	12.5	16	571	101
4	68	F	59 +/− 2	151 +/− 3	266	6.69	56.4	27.5	10.8	321	123
5	88	F	75 +/− 4	186 +/− 4	270	8.29	59.5	23.8	11.5	281	114
6	69	F	54 +/− 3	157 +/− 8	267	12.7	55.2	37.5	4.28	313	141
7	78	M	53 +/− 0	139 +/− 2	254	6.27	59	31.7	6.95	228	129
8	78	F	79 +/− 1	198 +/− 9	268	20.4	72.6	9.8	15.4	364	121
9	50	M	71 +/− 1	200 +/− 2	293	4.49	45.7	25.3	23.6	333	95.9
10	58	M	45 +/− 1	147 +/− 3	294	8.14	50.3	35.8	10.9	233	125
R^2	**0.053**		**0.851**	**1**	**0.194**	**0.064**	**0.001**	**0.088**	**0.188**	**0.016**	**0.216**

gH2AX 30 and 100 nM indicates the mean FITC intensity obtained in WBC after treatment of the blood with 30 and 100 nM CLM, respectively. WBC indicates the white blood cell count. NEU%, LYM%, MON% indicates the percentage of neutrophils, lymphocytes or monocytes respectively. In the differential count PLT indicates the platelet count. HGB indicates blood hemoglobin concentration. TSB indicates the percentage DNA damage remaining after 0.5 h at 37°C measured by the comet assay. R^2 indicates the linear coefficient of determination between gH2AX signal at 100 nM CLM and the indicated parameter.

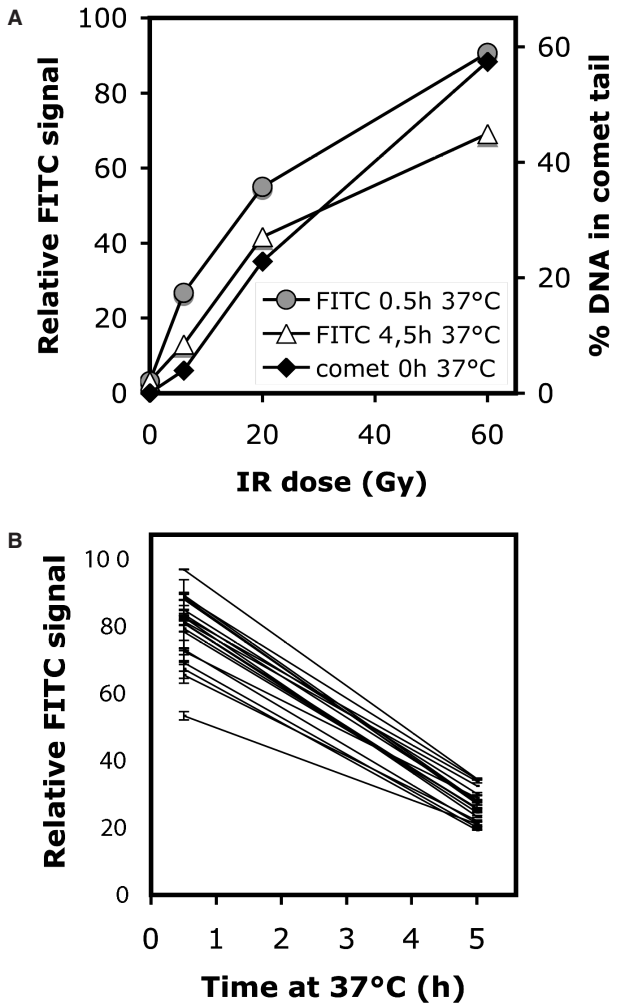

Figure 5. The gamma-H2AX signal in blood lymphocytes irradiated with gamma radiation. (**A**) Measurement of IR-induced DNA damage by the gamma-H2AX assay and the comet assay in lymphocytes. Blood samples from a healthy subject were irradiated on ice with 0, 6, 20 or 60 Gy of gamma radiation. Lymphocytes were prepared from a sample of each irradiated blood sample and analyzed by the comet assay. The irradiated blood samples were incubated for 0.5 or 4.5 h at 37°C to allow H2AX phosphorylation. Lymphocytes were prepared and analyzed with the gamma-H2AX assay. (**B**) The IR-induced gamma-H2AX signal in lymphocytes shows significant variation among individuals. Blood from 20 patients were irradiated on ice with 8 Gy of gamma radiation and incubated for 0, 0.5 or 5 h at 37°C before preparation of lymphocytes. The lymphocytes were analyzed by the gamma-H2AX assay and by the comet assay. Error bars represent the difference in mean FITC signal intensity between double samples.

significant correlation between the gamma-H2AX signal and other parameters analyzed (Table 2). We also analyzed the gamma-H2AX signal after *in vitro* CLM treatment of blood samples from nine children (1–16 years), and eleven patients around eighty years old (77–89 years). There was no statistical difference in the gamma-H2AX signal between these age groups (Figure 4B).

We also wanted to use IR to examine the interindividual difference in gamma-H2AX response. Therefore, to find the dynamic range of the gamma-H2AX signal in response to IR, we irradiated blood from one healthy subject with different doses of gamma radiation. The blood were kept on ice, or incubated at 37°C for different times. Lymphocytes were prepared and the resulting gamma-H2AX signal measured. The results indicated that there was a linear increase of the gamma-H2AX signal up to 20 Gy and that incubation for 30 min at 37°C gave an optimal gamma-H2AX signal (Figure 5A). We also noted that there was a reduction of the gamma-H2AX signal after 4.5 h incubation at 37°C that was more pronounced at low doses of IR, probably reflecting repair of DSBs (36). Essentially no reduction of gamma-H2AX was observed in granulocytes from the same blood samples (data not shown), indicating that measurement of DSB repair by gamma-H2AX removal must be done on purified lymphocytes. Based on this information we decided to irradiate blood from 20 patients with 8 Gy, a dose expected to be well within the linear dose range with respect to the gamma-H2AX signal. Lymphocytes were prepared directly after irradiation and analyzed by the comet assay to determine the initial amount of DNA damage (Table 3). The rest of the irradiated blood was divided into two samples. One sample was incubated for 30 min and the other for 5 h at 37°C before lymphocyte preparation and analysis with the gamma-H2AX assay and the comet assay. We observed a 2-fold difference in gamma-H2AX signal among the patients (Figure 5B). The gamma-H2AX signal correlated poorly with all blood parameters analyzed (Table 3).

Previous reports indicate that the rate of gamma-H2AX removal correlates with DSB repair capacity (37), and that this correlation is best observed at doses of IR around 2 Gy (36). Although the IR dose in this experiment was optimized to observe differences in the initial gamma-H2AX response we used gamma-H2AX signal reduction after 5 h as a marker for DSB repair in the patients. We found that the reduction in gamma-H2AX signal after 5 h varied between 59 and 71% among the patients (Figure 5B). This indicated that the variation of DSB repair capacity among the patients was in the order of 0.3-fold, compared to the 2-fold variation in initial gamma-H2AX signal. However, similar experiments using lower doses of IR must be performed to more accurately measure DSB repair rates.

In conclusion, we find that our flow-cytometry-based method conveniently allows measurement of the gamma-H2AX signal in routine patient samples and reveals a marked variation in the gamma-H2AX signal in blood cells exposed to CLM or IR among patients.

DISCUSSION

Gamma-H2AX (γH2AX) has been shown to be a sensitive indicator of DSBs produced by IR, drugs or physiological processes, such as V(D)J-recombination (11). Using flow cytometry, we have optimized a method for detecting gamma-H2AX in human blood cells. Since the DNA damage induced signal from the FITC-labeled monoclonal antibody was absent in H2AX−/− cells we

Table 3. Analysis of the gamma-H2AX signal in response to IR in twenty patients

Patient	Age	Sex	gH2AX after 0.5 h at 37°C	gH2AX after 5 h at 37°C	Comet (visual score)	WBC (10^9/l)	NEU%	LYM%	MON%	PLT (10^9/l)	HGB (g/l)	TSB repair (% of initial damage)
1	41	M	202 +/− 4	88 +/− 5	230	7.49	51.3	37.7	6.6	198	162	99.8
2	52	M	291 +/− 3	107 +/− 1	200	5.84	71.4	16	10.3	150	135	99.3
3	53	F	311 +/− 10	120 +/− 2	248	9.69	76.6	15.2	10.3	331	110	99.9
4	10	M	270 +/− 1	90 +/− 3	227	2.31	17.4	36.8	36.5	326	136	99.7
5	67	M	297 +/− 8	102 +/− 0	208	5.1	63.5	23.1	9.7	355	119	100
6	20	F	352 +/− 0	136 +/− 1	193	2.69	62.1	27.4	7.7	62.2	104	100
7	77	M	303 +/− 4	97 +/− 2	164	13.9	84.9	6.3	7.8	150	131	100
8	32	M	250 +/− 15	84 +/− 0	80	14	79.3	8	11.4	171	166	99.9
9	73	M	323 +/− 3	110 +/− 3	132	10.8	79.9	13.9	4.2	384	98	99.9
10	77	M	326 +/− 16	113 +/− 2	208	11.7	84.2	9.1	6.3	275	89	100
11	57	F	288 +/− 17	104 +/− 6	230	10.5	55.7	31.2	11.9	81	134	100
12	45	M	297 +/− 2	105 +/− 1	173	6.31	55.5	28.7	8.8	239	107	100
13	58	F	321 +/− 6	113 +/− 1	125	9.01	70	17.2	11.2	393	108	100
14	32	M	257 +/− 2	86 +/− 2	157	12.8	80.5	7.7	10.1	181	168	100
15	43	F	303 +/− 1	133 +/− 3	128	3.75	75.7	11.8	12.2	73.5	90.7	100
16	68	F	306 +/− 5	115 +/− 1	140	4.75	71.2	17.8	9.2	176	114	100
17	72	F	268 +/− 11	115 +/− 5	125	5.02	32.1	52	12.9	327	141	100
18	80	F	299 +/− 3	128 +/− 4	241	19.1	90.6	5.3	3.1	296	116	100
19	28	F	244 +/− 4	93 +/− 1	159	2.61	70.5	7.6	21.7	79.2	85.3	100
20	23	F	324 +/− 4	136 +/− 1	231	6.38	80.1	11.3	8.2	32.3	89.9	99.9
R^2	**0.02**		**1**	**0.2635**	**0.0024**	**0.005**	**0.0149**	**0.0041**	**0.1868**	**0.002**	**0.272**	**0.0507**

gH2AX after 0.5 and 5 h indicates the mean FITC intensity obtained in lymphocytes after irradiation and incubation at the indicated times at 37°C. WBC indicates the white blood cell count. NEU%, LYM%, MON% indicates the percentage of neutrophils, lymphocytes or monocytes respectively. In the differential count PLT indicates the platelet count. HGB indicates blood hemoglobin concentration. TSB indicates the percentage of the intitial DNA damage remaining after 0.5 h at 37°C measured by the comet assay. R^2 indicates the linear coefficient of determination between gH2AX signal after 0.5 h and the indicated parameter.

concluded that our assay specifically detects gamma-H2AX accumulation. Previous protocols for flow-cytometry measurement of gamma-H2AX have used fixed cells. We found that fixation could be omitted, and this significantly improved the gamma-H2AX staining intensity in WBC from patient blood samples. It is possible that less compact chromatin in non-fixed nuclei result in improved antigen presentation. The gamma-H2AX signal was linearly correlated with the level of strand breaks and allowed detection of strand breaks induced by CLM hundred times below the detection limit for the comet assay. The comet assay is a sensitive method that measures DNA strand breaks in individual cells. This assay is capable of detecting strand breaks in cells exposed to 1–2 Gy, close to the mean lethal dose. This shows that gamma-H2AX measurement on non-fixed cells is a very sensitive and simple way to measure DSB levels in human subjects.

We also found that blood samples could be stored on ice for extended periods of time without loss of the gamma-H2AX signal, which is important in the clinical setting where blood samples often are analyzed several hours to a day after they have been withdrawn. In addition, purified WBC or lymphocytes could be stored at −80°C for long periods of time without loss of the gamma-H2AX signal.

Gamma-H2AX can also be measured by immunohistochemistry. By counting gamma-H2AX- foci, it is possible to measure very low levels of DNA damage that are inflicted during routine X-ray examinations (38). A comparison of our flow-cytometry method with published data indicates that foci counting can detect close to ten times lower doses of IR (28). The gamma-H2AX foci method is, however, labor intensive, and will be hard to apply in clinical practice. In contrast, our flow cytometry method is currently in a format that can be used in routine flow cytometry analysis of patient samples.

Although our flow-cytometry method was optimized for cells obtained from patient blood, we have found that gamma-H2AX measurement in non-fixed cell lines result in lower background in non-damaged cells and higher gamma-H2AX signal in CLM-treated cells. Cell types that have been tested in this regard include primary MEFs (Figure 2), HeLa cells, primary human chondrocytes, EBV-transformed B-cells, glioma cells (Mo59K, Mo59J) and melanocytes (G361) (data not shown). Our method can therefore be used to measure gamma-H2AX induction in both clinical samples and in cells grown in the lab.

WBC prepared from normal individuals does not contain any cells in S- or G2-phase. We have therefore not included any measure of DNA content in the current form of the assay. It is however possible to add DNA-binding, fluorescent drugs, like Hoecht 33352 that does not overlap with FITC emission spectrum (34). However, efficient Hoecht 33352 detection requires expensive UV lasers, that most often are not available in routine flow cytometry machines. We found that the fluorescence signal from most other DNA dyes, including propidium iodide, partly overlap with the FITC signal from the gamma-H2AX-antibody and decreases the sensitivity of the assay especially at low levels of DNA damage. During the assay development, we observed that

the level of doublet nuclei and debris that contributed to the FITC signal from blood cells was very low. Therefore, we have not included any DNA dye in the gamma-H2AX assay.

To validate that our method could be used to monitor DSBs in patient cells, we used the gamma-H2AX assay to examine the DSB signal in response to CLM or IR in a small number of patients. We also measured the levels of strand breaks in the same population of cells, using the comet assay. The data indicated that there was a significant variation in the level of gamma-H2AX that did not correlate with the DNA damage level measured by the comet assay or patient age. We also failed to find any other routine blood parameter that correlated with the gamma-H2AX signal. It is possible that our finding represents an inherent difference in the gamma-H2AX signal among humans. This could, for instance, be due to difference in the number of gamma-H2AX foci formed at a given DNA damage level. This would imply that the efficiency by which the cell converts DSBs to gamma-H2AX foci varies among individuals. We find this explanation unlikely since the number of gamma-H2AX foci correlates well with the levels of DSBs measured with electrophoretic techniques (28) or by decay of ^{125}I incorporated in cellular DNA that produce DSBs with 90% efficiency (29). A more likely possibility is that the number of gamma-H2AX molecules produced per DSB varies among individuals. If this latter scenario were true, the interindividual variation of gamma-H2AX accumulation would not be possible to detect using gamma-H2AX foci counting since this method provides no information about the brightness of each focus. Regardless of the mechanism behind the gamma-H2AX signal variation, it will be interesting to examine if this phenomenon is linked to IR sensitivity (32–34) and therefore could be used to, as a part of radiotherapy planning, identify the most IR-sensitive patients (39).

Another interesting application of the gamma-H2AX assay would be to measure the DSB response in cancer patients undergoing treatment with DSB-inducing agents like IR, doxorubicin, etoposide or CLM-linked antibodies (gemtuzumab, ozogamicin). These drugs have a narrow therapeutic window and display variable pharmacokinetics. Therefore, the gamma-H2AX assay could allow us to administer DSB-inducing drugs based on the resulting DSB signal in the patient to allow individualized cancer treatment.

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