OPTIMISING IMMUNOSLOT BLOT ASSAYS AND APPLICATION TO LOW DNA ADDUCT LEVELS USING AN AMPLICATION APPROACH

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ABSTRACT

Immunoslot blot assays have been used for the analysis of many DNA adducts but problems are frequently encountered in achieving reproducible results. Each step of the assay has been systematically examined and it was found that the major problems are in the DNA fragmentation step and the use of the manifold apparatus. Optimisation was performed upon both the malondialdehyde-deoxyguanosine adduct (M_1dG) and the O^6-carboxymethyl-deoxyguanosine adduct (O^6CMdG) to demonstrate the applicability to other DNA adducts. Blood samples from the EPIC study (n = 162) were analysed for M_1dG adducts and the data showed no correlation with adduct levels in other tissues indicating that the EPIC blood samples were not useful for studying M_1dG adducts. Blood samples from a processed meat vs vegetarian diet intervention (n = 6) were analysed for O^6CMdG and many were below the limit of detection. The reduction of background adduct levels in standard DNA was investigated using chemical and whole-genome amplification approaches. The latter gave a sensitivity improvement of 2.6 adducts per 10^7 nucleotides for the analysis of O^6CMdG. Subsequent reanalysis for O^6CMdG showed a weakly significant increase in O^6CMdG on the processed meat diet compared with the vegetarian diet, demonstrating that further studies are warranted.

Keywords: immunoslot blot assay; DNA adducts; O^6CMdG; M_1dG; genome amplification; EPIC; processed meat

INTRODUCTION

Exposure of cellular DNA to exogenous and endogenous genotoxic agents results in a variety of DNA modifications, e.g. DNA adducts, which could be potentially mutagenic and represent an initiation step for carcinogenesis. The identification and quantification of very low concentrations of DNA lesions in vivo in multi-cell samples requires ultra-sensitive methodologies. This is true particularly for the analysis of human samples, where small amounts of sample and therefore DNA is normally available. The immunoslot blot (ISB) assay was first developed by Nehls and coworkers in 1984 (1). Since then the assay has been applied to the detection of several DNA adducts formed both in vitro (2-5) and in vivo (6-12) and the use of both monoclonal (13, 14) and polyclonal (2) antibodies has been described. Whilst the former are more specific, the latter tend to have greater sensitivity. In a previous paper the development of a very sensitive ISB assay was described for the detection of the endogenous adduct malondialdehyde-deoxyguanosine (M_1dG, Fig. 1), derived from lipid peroxidation, in small amounts of human white blood cell and gastric biopsy DNA and the potential for its use in human biomonitoring studies (15) using a monoclonal antibody (16).

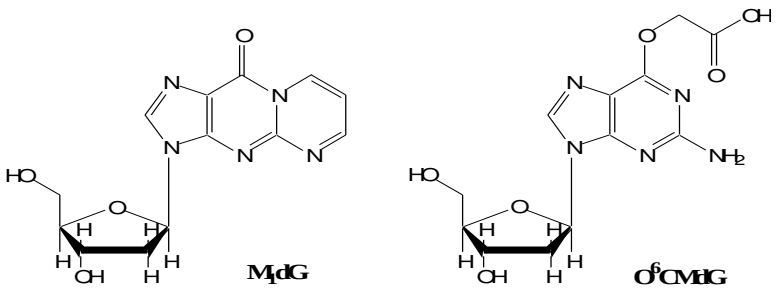

Fig. 1 Structures of the DNA adducts, M_1dG and O^6CMdG

The ISB has since been used to detect the M_1dG adducts in mice livers (17) and human colorectal mucosa (18) and more recently we applied this methodology to the measurement of O^6-carboxymethyl-deoxyguanosine adducts (O^6CMdG, Fig. 1), using a polyclonal antibody (19), derived from nitrosated amines, for in vitro studies and in human blood

samples of volunteers consuming a high red meat diet (20). The latter was confirmed by alternative qualitative methods of measuring O^6CMdG (21).

The advantage of the ISB assay over many other methods of measuring DNA damage is that it only requires small amounts of DNA (1 μg per sample) and replicates can be analysed on the same blot. The handling of radioactive compounds for methods such as ^{32}P-postlabelling is eliminated and a higher throughput of samples can be achieved, up to 18 samples in two days. However, the assay has many steps and problems can occur at any point; during fragmentation and denaturation of the double-stranded (DS) DNA standards and samples, immobilisation of the resulting single-stranded (SS) DNA on a nitrocellulose membrane, incubation with the primary antibody followed by incubation with a secondary antibody tagged with an enzyme complex such as horseradish peroxidase, treatment with a chemiluminescence reagent and finally in the acquirement of the chemiluminescence image. These problems have deterred researchers and the literature reveals relatively few studies that have used the ISB methodology. This study sought to identify, and provide solutions to, the most important factors in achieving reliable data from the ISB. Furthermore, a prerequisite for the ISB assay is the availability of an appropriate standard, for example calf thymus (CT) DNA containing known amounts of modification, in order to generate calibration lines on the blot. However, a common problem of measuring endogenous DNA adducts is the presence of background levels of the lesion of interest in untreated CT-DNA. This means that the detection limit of the assay is potentially lower than the actual limit set by the standard DNA. The sensitivity of the assay was sought to be improved either by elimination of the adduct in standard CT-DNA or by masking it such that it is not recognised by the primary antibody. The use of chemical modification and whole genome amplification approaches are described.

In addition, an interlab comparison was carried out and the analysis of blood samples was investigated as it was found that adducts levels in blood can be very low and thus difficult to

measure. The analysis of blood samples is particularly relevant for biomonitoring studies if they are to be as non-invasive as possible. The results for M_1dG in colorectal mucosa samples from the EPIC study had been previously reported and a weak correlation was found with dietary saturated fat (18) which was corroborated in an intervention study using blood samples (22). The corresponding EPIC blood samples have now been analysed and the results are reported in this paper. The analysis of O^6CMdG in blood was also investigated in volunteers that had consumed high levels of processed (nitrite-preserved red) meat compared to a vegetarian diet in a small scale pilot study. Previous research had identified the presence of the O^6CMdG adduct in volunteers consuming a high red meat diet (20) and established that processed meat leads to higher levels of DNA damage than red meat (23). Thus, the present study sought to identify differences in O^6CMdG adducts between a diet high in processed meat compared to a vegetarian diet.

MATERIALS & METHODS

Tetramethoxypropane (TMP), guanine, 2'-deoxyadenosine, 2'-deoxyadenosine-3'-monophosphate, CT–DNA (D1501), genomic ultrapure CT-DNA (D4764), propidium iodide (PI), micrococcal nuclease (MN), nuclease P1 (NP1) and goat anti-rabbit IgG horseradish peroxidase conjugate were obtained from Sigma-Aldrich (Dorset, UK). Calf spleen phosphodiesterase (CSPDE) and human genomic DNA (#1691112) were purchased from Boehringer Mannheim (Lewes, East Sussex, UK). Goat anti-mouse IgG horseradish peroxidase conjugate was purchased from Dako (Denmark). Phosphate buffered saline (PBS) tablets were purchased from Oxoid Ltd. (Basingstoke, UK). Qiagen genomic-tips 100/G were purchased from Qiagen Ltd (Crawley, UK). GenomiPhi™ V2 DNA Amplification Kits were purchased from GE Healthcare (Little Chalfont, UK). Methanol used for HPLC analysis was for fluorescence application and purchased from Fisher. All other reagents and solvents of

analytical grade or HPLC grade were obtained from Fisher Scientific Ltd. (Loughborough, UK) or Sigma-Aldrich.

Synthesis of analytical standards

M_1G (17), M_1dG (15), and O^6CMdG (19) were prepared as previously described. O^6CMG was obtained via acid hydrolysis of O^6CMdG in 0.1 M formic acid at 70º C for 1 h.

Preparation of M_1dG-DNA standards

Two highly modified M_1dG-DNA standards were prepared independently in different laboratories as previously described by treating CT-DNA with malondialdehyde (MDA) (15). The DNA concentration and purity were determined by UV spectroscopy using the absorbance at 260 nm and the A_{260}/A_{280} ratio on either a GeneQuant II RNA/DNA calculator (Pharmacia Biotech) or a Uvikon XL (Biotek) with LabPowerJ DNARNA purity check software. DNA was digested by either acid hydrolysis (see below) or enzymatic digestion (15) to the free purines or deoxynucleotides and deoxynucleosides respectively and the DNA concentration and adduct levels quantified by HPLC on a Waters Alliance system equipped with a Waters 996 photodiode array detector and a Waters 474 scanning fluorescent detector or a Waters 600E or Waters 2690 system equipped with a Waters 484 UV and a Waters 470 fluorescence detector with a narrow-bore Hypersil BDS C18 column (3 µm, 100 x 2.1 mm) including a prefilter. The digested DNA was analysed in triplicate (10 µl/sample) using an isocratic flow rate of 0.2 ml/min with 0.1 M triethylammonium acetate (pH 5.0), 1% methanol for M_1G or 4% methanol for M_1dG with fluorescence detection (λ_{Ex} 360 nm, λ_{Em} 500 nm). Normal bases were analysed at 260 nm by UV. The modified DNA was diluted to a DNA concentration of 100 µg/ml and an M_1dG concentration of 10 fmol/µg DNA with unmodified CT-DNA. Further dilutions were made with 100 µg/ml unmodified CT-DNA to produce standards in the range of 0-10 fmol M_1dG/µg DNA for the ISB calibration line.

Preparation of O^6CMdG-DNA standards

Highly modified O^6CMdG-DNA standards were prepared as described previously (20) by treating CT-DNA with potassium diazoacetate (KDA). O^6CMdG-DNA was analysed by HPLC as described above using a water/methanol gradient (70:5 to 40:35 in 15 mins) and 25% 0.1 M heptafluorobutyric acid, with UV analysis for normal bases (260 nm) and fluorescence detection of O^6CMdG and O^6CMG (λ_{Ex} 286 nm, λ_{Em} 378 nm). Quantification and preparation of standards for the ISB assay was as described above to give standards in the range 0-10 fmol O^6CMdG/µg DNA and 100 µg/ml DNA.

Optimisation of adduct-DNA acid hydrolysis

M_1dG-DNA was incubated with 0.1 M and 1 M formic acid or 0.1 M and 1 M HCl at 70° C for 1 h, and 0.1 M formic acid or 0.01-6 M HCl at 100° C for 1 h (24) using 2 µg M_1dG-DNA in 40 µl solution. The solutions were evaporated to dryness and redissolved in 40 µl of 1 mM formic acid or 1 mM HCl.

O^6CMdG-DNA (20 µg) was incubated with 0.1 M formic acid at 70° C for 1 h (17), 0.1 M HCl at 100° C for 30 min, 1 M TFA at RT or 50° C for 1 h (25), and 1 M acetic acid at 50° C for 3 h, and then neutralised with 0.1 M NaOH, and evaporated to dryness and redissolved in 40 µl 0.1% HFBA.

Optimisation of the immunoslot blot assay

The ISB assay was developed from a previous method (15) with modifications to the original procedure to improve DNA binding (see below) to the nitrocellulose (NC) membrane (0.1 µm, BA79, or 0.45 µm, BA85 Schleicher & Schuell, Dassel, Germany). The primary antibody concentrations were: anti-M_1dG monoclonal antibody D10A1 (16) diluted 1:90,000, and anti-O^6CMdG polyclonal antibody (19) diluted 1:800. The secondary antibody concentrations were: goat anti-mouse IgG horseradish peroxidase conjugate diluted 1:4,000,

and goat anti-rabbit IgG horseradish peroxidase conjugate diluted 1:2,000. Enzymatic activity was visualised by bathing the membranes for 5 min in Supersignal Ultra (Pierce). Chemiluminescence signals from the NC membrane were captured using either the Biorad Fluor STM Multimager with the Multianalyst software (Biorad) or on the Kodak Image Station 440CF with correction for local background.

Comparison of sonication methods

DNA fragmentation was investigated for a number of different sonicators and times: DNA samples were 1) sonicated in a Ultrawave U100H and Fisherbrand FB100 bath (2-20 mins followed by heat-denaturation at 100º C for 10 min and then kept on ice for 10 min; 2) only heat-denatured (no sonication); 3) heat-denatured first and then sonicated; 4) sonications were carried out on ice. DNA fragmentation was monitored either by gel electrophoresis or by binding to the NC membranes as measured by PI staining.

Improvement of DNA binding to nitrocellulose membrane

The following conditions were examined in order to improve DNA binding to NC membranes and assessed using the ISB and PI assays: 1) Membranes were baked in a vacuum oven at 80° C for 1.5 h (1), 2) NC membranes were bathed for 5 min in SSC buffer (0.75 M NaCl, 0.075 M trisodium citrate, pH 7.0) prior to baking, 3) Effects of sample temperature: samples were either removed from the icebath after completion of the denaturation step or kept at 4° C until application to the membrane, 4) decreasing the vacuum applied during sample aspiration and 5) adjustment of the manifold apparatus (Minifold II, 72 well slot blot microfiltration apparatus (Schleicher & Schuell)) via insertion of additional spacers.

Agarose gel electrophoresis

A volume equivalent to 1 µg DNA or 1 µl of marker were mixed with 2 µl of gel loading solution (type 1, 6x concentrate, Sigma G-7654) and made up to 10 µl with ultrapure water.

A 2% agarose gel containing ethidium bromide (2.0 µl/100 ml gel) (EtBr, 10 mg/ml) was run in 1xTBE buffer (working solution: 90 mM Tris-borate, 2 mM EDTA; pH 8.0) at 120 V for 1.75 h. A 100 basepair (bp) DNA ladder (Life Technologies, No. 15628-019, 0.25 µg/µl) was used as a marker (fragment sizes: 2072, 1500, 1400, 200, 100 bp). The gel image was captured using the Biorad Fluor-S^{TM} MultiImager.

Quantification of DNA binding by propidium iodide staining

Two CT-DNA calibration lines (0.10-2.50 µg DNA/well) were prepared on the same membrane. One half was washed twice for 10 min in 50 ml PBS and the other overnight and then both were stained with PI for 3 h. Variations in DNA binding of different sources of DNA were examined by comparison of CT-DNA, M_1dG-DNA, O^6CMdG-DNA, Boehringer Mannheim (BM) standard DNA and human WBC DNA diluted to give 0.1-2.5 µg DNA/well and adduct levels (where appropriate) of 0-10 fmol adduct/µg DNA. The ISB procedure was followed and the membranes incubated overnight with PI following a 10 min wash. All membranes were washed for 1 h in PBS (50 ml) prior to capture of the fluorescent signal using either Biorad Fluor-S^{TM} MultiImager or Kodak Imager with filters for >520 nm.

Reduction of M_1dG adduct levels by chemical modification

Highly modified M_1dG-DNA solutions (50 µg, 18 pmol M_1dG/µg DNA) were dissolved in KP buffer (10 mM K_2HPO_4, pH 8, 8.5 and 9) and treated with methoxyamine (5, 20 and 50 mM). The samples were incubated at -20° C for 16-66 h and then 37° C. Aliquots (4 µg) were taken at 0-24 h, enzymatically digested to the deoxynucleosides and analysed by HPLC.

Unmodified CT-DNA (350 µg) was dissolved in KP buffer, pH 8 and treated with 0-5 mM methoxyamine, incubated for 16-94 h at -20° C followed by 0-24 h incubation at 37° C. DNA was precipitated with 0.8 vol isopropanol, washed with 70% ethanol and redissolved in water.

CT-DNA samples were analysed by immunnodot blot (IDB) with PI correction and the adduct levels calculated from calibration lines acquired with the unmodified CT-DNA.

Reduction of adduct levels by DNA amplification

10 ng CT-DNA, M_1dG-DNA (21 pmol M_1dG/ug DNA) O^6CMdG-DNA (1.3 pmol O^6CMdG/ug DNA) and the kit control were amplified using a GenomiPhi™ kit following the manufacturer's protocol. The DNA was purified by precipitation with a sodium acetate/EDTA buffer (final concentration = 0.25 M/0.04 M, pH 8.0) and quantified by UV. The adduct levels in the amplified DNA samples were quantified by ISB using the original standards. Calibration lines were compared for the amplified and original CT-DNA with standards in the range 0-3 fmol/ug DNA.

Human blood samples for M_1dG analysis

162 blood samples from the EPIC study that had been collected at the same time as colon biopsy samples (18) were analysed for M_1dG adducts.

Human blood samples for O^6CMdG analysis

Healthy males (n = 4) and females (n = 2) from Cambridgeshire were recruited through local advertisements and participated in a randomised crossover intervention study consuming processed meat versus vegetarian diets as described previously (23). The participants were between 25 and 50 years of age, nonsmokers, free from diabetes and bowel disease, not taking medication affecting the gut for at least 3 months prior to the study, not pregnant and not participating in another biochemical intervention study at the same time. The studies were approved by the Cambridge Local Research Ethics Committee. Blood samples were collected at the end of each dietary period and the DNA isolated using Qiagen kits.

Analysis of blood samples

All blood samples were analysed using the optimised ISB assay on a 0.1 µm membrane with PI correction. Calibration lines were in the range of 0-5 fmol adducts/µg DNA using either CT-DNA or amplified CT-DNA to produce the standards. Analyses were performed in triplicate on the same blot and only the results with a SD of < 20% were included in the reported data. A human DNA QC sample (Boehringer Mannheim) was included in all assays for M_1dG and the results rejected if the QC had a SD of > 20% for the triplicate analyses or the QC result was > 2 SD from the mean of all the QC results (2.00 adducts per 10^7 normal bases +/- sd 1.27). The limit of detection was 0.2 adducts per 10^7 normal nucleotides (15).

Statistics

Statistical analyses were performed using SPSS for Windows version 15.0. Correlations between M_1dG adduct levels in blood and colon biopsies were assessed by Spearman rank correlation coefficient. Differences in O^6CMdG adduct levels between dietary periods were assessed by Wilcoxon signed rank tests for related samples.

RESULTS

Preparation of standards for ISB assays

Under mild acid hydrolysis conditions only the purine bases are completely released. Acid hydrolysis of DNA samples was not found to affect the guanine:adenine ratio under any of conditions tested. However, the ratio for M_1G:G varied considerably; incubation with 0.1 M formic acid at 70° C released the highest level of adduct (M_1dG:G 0.13:1) whereas 0.01-6 M HCl at 70-100° C resulted in reduced adduct levels, and release of pyrimidine bases in the highest acid concentrations. Similarly, 0.1 M HCl gave a reduced level of O^6CMG adducts relative to 0.1 M formic acid. Hydrolysis of O^6CMdG with 1 M TFA (25) was not as effective as 0.1 M formic acid as was evident from the presence of a small peak for O^6CMdG

in the chromatogram of the hydrolysed sample. Hence, 0.1 M formic acid was determined to be the best method for hydrolysis with respect to both DNA concentration and adduct quantitation for both M_1dG and O^6CMdG.

Two M_1dG-DNA standards were prepared and analysed in independent labs by different methods. In lab 1 the DNA was hydrolysed using the 0.1 M formic acid method at 70° C and the M_1dG adduct level was determined to be 21 pmol/µg DNA. Lab 2 prepared separate standards and determined the M_1dG adduct level to be 18 pmol/µg which was confirmed by an LC-MS method in Nashville, USA where it was established as approximately 17 pmol/µg by John Plastaras (personal communication). Comparison of the two diluted standards by ISB assay gave very similar results for the calibration lines thus validating the methods used for preparation of standards and acquirement of calibration lines by independent labs; Lab 1: R^2 = 0.95, y = 43862x + 45004, Lab 2: R^2 = 0.99, y = 38579x + 28721.

Quantitation of DNA binding by propidium iodide staining

The effects of residual reagents upon PI staining were investigated and the fluorescent signals were similar for an initial wash time of 10 min and overnight (data not shown). CT-DNA calibration lines were acquired with a PI incubation time of overnight or 3 h and both gave a good correlation between the amount of DNA bound to the membrane and the signal intensity (R^2 = 0.95 and 0.98). However, the shorter incubation time gave the better correlation and a higher slope (3304 compared with 1864). The adduct levels were not found to affect the PI intensity in either the M_1dG-DNA or O^6CMdG-DNA standards as compared with the CT-DNA standards (data not shown)

DNA fragmentation and improvement of DNA binding to NC membranes

The PI assay revealed major differences in binding to NC membrane between human and CT DNA, 1.5-2.5 times more human WBC DNA was binding to the NC membrane as compared

to the CT-DNA standards. DNA quantitation for samples and standards was reliable (UV and HPLC analysis) so the cause must be due to one of the sample preparation steps. The fragmentation of human WBC and CT-DNA following sonication was investigated using agarose gel electrophoresis. Differences in fragmentation between CT-DNA and human WBC DNA were quite evident; in general CT-DNA fragmented to lower molecular weight fragments (100-600 bp) than human genomic DNA (fragments >600 bp). Ultrapure CT-DNA (Sigma) gave fragments greater than 600 bp.

The PI staining showed that a reduction in sonication times and sonication on ice were both beneficial to reducing the variability in DNA binding to NC membranes whereas the order, or omission, of the sonication and denaturation steps had no effect upon the relative binding of different DNA sources. Comparison of the Fisherbrand and Ultrawave sonication baths also revealed major differences in DNA binding. Previous papers reported sonication times of 20 min but 2 min sonication on ice gave a poor calibration line with the Fisherbrand sonicator whereas very good calibration lines could be obtained with the Ultrawave sonicator. Hence, fragment size is very dependent upon the sonicator used and the sonication time. Sonication is known to be a variable method for sample preparation but can be used under carefully controlled conditions that need to be verified prior to the ISB assay. Furthermore it is far more time and cost effective than enzymatic hydrolysis.

Further improvement in binding DNA was achieved by keeping the samples on ice after the denaturation step until application to the NC membrane (CV = 13%). The PI signals for CT-DNA and BM DNA were also much closer in intensity, with only 12% and 10% variation within each sample set, and a 21% variation overall. Substituting ammonium acetate for 20x SSC buffer made no difference to the DNA binding or quality of calibration lines obtained (R^2 = 0.99 for both buffers).

The binding of DNA on two different NC membranes was investigated using the PI assay. Only 33% of DNA was binding to the 0.45µm membrane compared to the 0.1 µm membrane. Baking the membrane at 80° C for 1.5 h in a vacuum oven compared to a normal oven resulted in approximately 40% more DNA bound. When the NC membrane was bathed in SSC buffer, 44% more DNA was binding to the membrane. However, relative differences in binding to the membrane between CT-DNA and human samples, as measured by PI ratios, did not alter using either method.

A critical factor appeared to be the ISB manifold. An increase in the tightness of the manifold apparatus by insertion of additional spacers in conjunction with a reduced vacuum during aspiration gave more consistent binding across the entire membrane as seen by the variation in PI intensity. The CV for the PI signal across the entire plate was improved from 26% to 15% with a reduction in vacuum from 60 to 0.5 mm Hg and the insertion of three acetate layers as additional spacers.

Reduction of M_1dG Levels by chemical modification

Background adduct levels in DNA can in principal be reduced by chemical modification such that the primary antibody does not recognise it. Niedernhofer et al (26) discovered that raising the pH causes the M_1dG adduct ring to open. This was utilised for subsequent reaction with methoxyamine to occur. M_1dG levels were reduced in highly modified M_1dG-DNA following treatment with 5 mM methoxyamine in KP buffer at different pHs. Incubating the samples for 16-66 h at -20° C showed a progressive reduction to 42-76% of the original value which reduced further with incubation at 37° C (an additional 12% at pH 8.0 and 17% at pH 9.0). No adverse effect was observed for normal nucleotides whereas treatment with higher concentrations of methoxyamine gave a 20-50% decrease in dAp. We also investigated the modification with hydroxylamine (data not shown) but this gave less reliable results than the methoxyamine experiments. Analysis of methoxyamine treated CT-DNA by IDB assay

showed that there was a reduction in background adduct levels for all the treatments (data not shown) compared with the untreated CT-DNA. This finding agreed with the HPLC results for the highly modified M_1dG-DNA. However, use of the methoxyamine-treated CT-DNA to prepare standards for the ISB assay gave no benefit in terms of a reduced background signal for the ISB calibration line

Reduction of background adduct levels by whole genome amplification

Amplification of DNA using the GenomiPhi™ kits, followed by purification, gave good yields of high quality DNA. Initial experiments with highly modified O^6CMdG-DNA showed that the adduct levels could be reduced to almost zero or below zero relative to the unmodified CT-DNA used for the ISB standards (Table 1). This shows a reduction in adduct levels of almost three orders of magnitude i.e. 1 pmol to 2 fmol. Other sources of DNA, which should be close to baseline values, all gave adduct levels below the LOD of the ISB assay after amplification (Table 1). Amplification of CT-DNA was then performed and the amplified DNA used to produce standards for the ISB assay by mixing it with the highly modified standard DNA for either M_1dG or O^6CMdG. The calibration lines all had good linearity (R^2 >0.97), however, the O^6CMdG assay gave a lower background reading as indicated by the intercept (2008 compared to 4150 for normal CT-DNA) as was to be expected if the adducts had been diluted by the amplification process but the M_1dG assay actually gave higher background readings. This was verified in triplicate thus the background for M_1dG is not due solely to adducts in the CT-DNA, but possibly non-specific binding or interference from other aspects of the assay. Therefore DNA amplification is not beneficial in the case of the M_1dG calibration lines but has proven beneficial for O^6CMdG assay.

Table 1 O^6CMdG adduct levels in amplified DNA measured by ISB (mean ± sd) relative to the original CT-DNA standards. The amplification reactions for each sample were prepared and performed in triplicate.

Amplified DNA sample	**O^6CMdG levels (fmol/ µg DNA) ± sd**
Unmodified CT DNA	-0.18 ± 2.10
Highly modified O^6CMdG-DNA	2.33 ± 3.18
human DNA	-0.95 ± 1.09
Genomiphi kit control DNA	-0.57 ± 0.65

M_1dG adducts in blood samples from the EPIC study

162 blood samples were analysed for M_1dG adducts and had an average of 1.61 (sd 0.59), adducts per 10^7 normal nucleotides (range 0.36-3.79). These were much lower than previously found in the biopsy samples where average levels were 4.45 (sd 2.99) adducts per 10^7 (range 0-11.89). The correlation coefficient between the two sets of samples was 0.06, p = 0.55. Some of the samples were at, or below, the LOD but as the amplification approach had proved unsuccessful for M_1dG it was not possible to perform any further analyses.

O^6CMdG adducts in blood samples from a processed meat study

Blood samples were analysed using the optimised ISB procedure but all samples, with one exception, had O^6CMdG levels below the LOD using standards produced from CT-DNA. Calibration curves were then produced with the amplified DNA and highly modified CT-DNA and these samples were reanalysed on a single blot. All samples gave a positive result with the amplified DNA calibration line whereas only one sample had a positive result with the conventional CT-DNA calibration line. The mean results are shown in Table 2 whilst Fig. 2 shows that the general trend for each volunteer was a higher adduct level on the processed meat diet compared with the vegetarian diet. The data was analysed by Wilcoxon signed

ranks test and gave a weak significant difference between processed meat and vegetarian diets (Z = -1.782, p = 0.075). The use of amplified DNA for preparation of standards gave an increase in sensitivity of 2.6 adducts per 10^7 nucleotides and the results presented show the ability of the technique to differentiate between samples at this level.

Table 2 O6CMdG adduct levels in human blood DNA from volunteers on a processed meat vs vegetarian diet. Adduct analysis was by ISB using standards prepared from amplified DNA with correction by PI staining, and statistical analysis by Wilcoxon signed ranks test.

	Processed meat	**Vegetarian**
Mean	1.84	1.51
Range	1.43-2.63	1.29-1.70
SD	0.43	0.17
Z = -1.782		p = 0.075

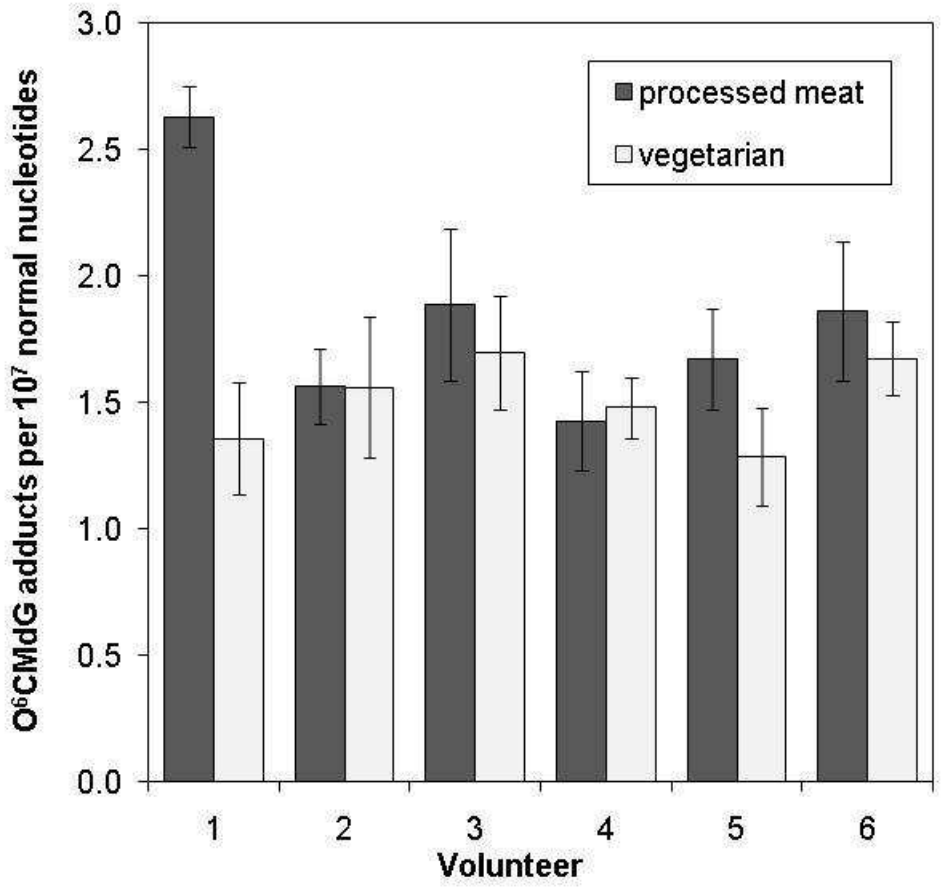

Fig. 2 O^6CMdG adduct levels (mean ± sd, CV = 4-20%) in blood samples from volunteers consuming processed meat vs vegetarian diets. Adduct levels were measured by ISB using calibration lines produced from amplified DNA.

DISCUSSION

When performing any analytical measurements it is essential to have a reliable and accurately quantified standard. We have shown that it is possible to quantify a M_1dG-DNA standard by a number of methods which included an acid hydrolysis step that needed to be optimised for the adduct recovery. The calibration lines compared well with those using enzymatic cleavage from a different lab. Having obtained and quantified a reliable standard we investigated all aspects of the ISB assay. The model of ultrasonic bath did have an effect upon DNA binding to the membranes due to the difference in fragmentation that occurred. A reduction in sonication time lead to improved binding due to larger DNA fragments being

obtained, as would be expected. Fragmentation is usually achieved by enzymatic methods but is more time consuming and costly and, therefore sonication is preferable if the reproducibility can be assured. Performing the sonication on ice resulted in further improvements to DNA binding presumably due to preventing the formation of superheated microcavities which may cause localised degradation of the DNA structure to give a number of fragments that are too small to be retained upon the NC membrane. The critical factor for a successful ISB assay is binding of the DNA to the NC membrane. As expected a smaller pore size led to greater binding of DNA and the 0.1 µm membrane is now used for all ISB analyses in our group. The PI assay had revealed that DNA binding was often higher on the underside of the membrane. One reason could be due to too high a vacuum pulling the DNA through the membrane too quickly and preventing efficient binding. A water aspirator is typically used for the vacuum and gives around 60 mm Hg. Other groups state that a moderate vacuum was used but do not specify the actual vacuum. The use of a vacuum pump allowed the vacuum to be reduced to a level that gave improved binding. The best result was achieved with a very low vacuum (0-10 mm Hg). Another factor was the tightness of the minifold apparatus as the design does not allow the manifold to be tightened sufficiently. The insertion of additional spacers helped to prevent bleeding of the DNA from the wells. This is a matter that can only be resolved by trial and error as each manifold apparatus will vary from the next and will alter with time.

DNA binding to NC membranes is greater for SS-DNA than DS-DNA due to interactions between the negatively charged backbone and the membrane surface. It is known that cations such as Mg^{2+} can decrease DNA binding (27) but as we used ultrapure water throughout the study this was not an issue. The protocol for the ISB assay includes a heat denaturation step followed by cooling on ice. However, the samples need to be removed for subsequent steps such as centrifugation and addition of reagents which may result in reannealing of DNA. It is

therefore essential to keep the samples as cold as possible until application to the membrane to ensure maximum DNA binding. The combination of sonication on ice and keeping samples on ice until application to the membrane gave much improved DNA binding, which resulted in improved calibration lines and lower CVs for triplicate analyses. It is possible to use DS-DNA that has not been fragmented but this results in a reduced chemiluminescence signal even when the quantity of DNA matches that of SS-DNA. This is presumably due to steric hindrance preventing access to the adduct by the antibody. TEXT DELETED

The reduction of background levels of M_1dG adducts with methoxyamine had looked promising initially. However, there were still results where the samples had a lower chemiluminescence signal than the bottom standard which should not contain any M_1dG adducts. An alternative approach using the whole genome amplication technique was investigated but still did not give a reduced background signal for M_1dG. However, a reduction in background levels of adducts using the whole genome amplification approach was successful with O^6CMdG. Furthermore, blood samples that had previously been below the limit of detection were reanalysed using the amplified DNA to prepare calibration lines and positive results obtained. Thus, this method may prove useful where adduct levels are expected to be low and a high sensitivity is required such as in the case of blood samples. However, as the background signal, and hence sensitivity, was not reduced in the case of M_1dG, this method is not suitable for all adducts. The difference between antibodies to the amplified DNA may be due to the monoclonal (M_1dG) being more specific than the polyclonal (O^6CMdG), and thus the whole genome amplification approach is more applicable to reduction of background signals where polyclonal antibodies are in use.

The current analysis of M_1dG levels in blood samples from participants in the EPIC study showed no correlation to earlier results from colorectal samples. However, an intervention study had demonstrated the importance of taking samples at appropriate time points (22).

This data indicates that the blood samples may not be useful in the case of M_1dG due to the timing of samples from the EPIC study but the analysis of urine samples by LC-MS may yield more informative data (28). The analysis of blood samples from the processed meat study did show a difference in adduct levels compared to the vegetarian diet for the majority of subjects with no difference between the sexes. This is the first case of a difference having been found between diets for O^6CMdG adducts and warrants further study to investigate the link between red meat or processed meat and colorectal cancer. In particular, the timing of samples and correlation with other tissues would need to be verified before application to large scale studies such as EPIC. The study would need to be repeated on a larger scale in order to have sufficient power to show a significant difference. Clearly, the adduct levels were very low and the background signal needs to be reduced using a technique such as whole genome amplification for the production of standards.

CONCLUSIONS

We found that the combination of many factors was synergistic in effect and led to a level of improvement for the ISB assay not seen for a single factor alone. The most important factors were the careful control of the fragmentation step if sonication methods were used and the manifold vacuum, both of which must be determined experimentally for each set of laboratory equipment. The ISB assay has been investigated thoroughly and is now at a point where good data can be obtained, even from samples with very low adduct levels such as blood. Chemical modification and genome amplification of untreated CT-DNA did not improve sensitivity for M_1dG but standards produced from whole genome amplification may be beneficial in improving assay sensitivity for other adducts such as O^6CMdG. The samples analysed for M_1dG have shown that blood DNA may not be a good choice for biomonitoring in studies such as EPIC as the timing of sample collection is likely to have been critical. However, blood samples may be useful for studying other adducts, such as O^6CMdG and

colorectal cancer risk, once the adducts have been proven to be a good indicator of disease risk through large scale studies where timing of samples has been thoroughly investigated.

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