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Further Evidence for a Possible Role of Conformation in the Immunogenicity and Antigenicity of the Oxidative DNA Lesion, 8-Oxo-2′Deoxyguanosine

MARCUS S. COOKE*, KARL E. HERBERT, PAUL C. BUTLER and JOSEPH LUNEC

Division of Chemical Pathology, Centre for Mechanisms of Human Toxicity, University of Leicester, The Hodgkin Building, P.O. Box 138, Lancaster Road, Leicester LE1 9HN, UK

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Damage to DNA by reactive oxygen species is acknowledged to be an important factor in a number of pathological conditions, including ageing and carcinogenesis. As a consequence, the development of methods for the sensitive detection and quantitation of oxidative DNA lesions has been of paramount importance. The oxidatively modified base product which has achieved most attention is 8-oxodeoxyguanosine (8-oxodG) and is a recognised marker of oxidative DNA damage.

Although both polyclonal and monoclonal antibodies have previously been raised to 8-oxodG these have, for the most part, failed to recognise this lesion within the DNA polymer. We have, through dilution cloning, produced a monoclonal antibody which appears to preferentially recognise 8-oxodG over deoxyguanosine (dG) in single-stranded oxidatively modified DNA. Such discrimination was not apparent when the DNA was double-stranded. Previous work has shown that 8-oxodG favours the *syn* glycosidic conformation due to steric repulsion, whereas dG assumes the *anti*. We present initial data that appear to support the postulate that it is these differences in conformation, in addition to structural recognition of the lesion itself, which are responsible for the discrimination, by our antibody, of 8-oxodG over dG in single-stranded DNA.

Keywords: ROS, 8-oxo-2′deoxyguanosine, antibodies, ELISA, DNA

INTRODUCTION

A role for free radicals and reactive oxygen species (ROS) has been proposed in a wide range of pathological conditions including, ageing,[1] inflammation,[2] autoimmunity[3] and carcinogenesis.[4] Although the product of normal cellular metabolism, ROS may damage cellular constituents with potentially deleterious results. DNA represents an important target with which ROS may interact, giving rise to variety of base and sugar products, strand breaks and protein crosslinks[5] which may lead to mutation or cell death. Of all oxidative base products, the one most studied is 8-oxoguanine (8-oxoG). This lesion has been reported to be elevated in conditions of oxidative stress[6] and also give rise to

*Corresponding author. Tel.: (0116) 2525590. Fax: (0116) 2525593. E-mail: MSC5@le.ac.uk.

mutations, which include G→C and A→C substitutions.

In order to fully assess the role of this lesion in pathology it is important to be able to quantitate levels of this lesion, both in DNA and in biological samples. A number of methods have been developed to achieve this, primarily HPLC with electrochemical detection (ECD)[7] and gas chromatography–mass spectrometry.[8] However, these methods require extraction and hydrolysis of the DNA, which, as part of sample workup, may induce artefactual damage.[9] Furthermore, using chromatographic methods, localisation of the damage *in situ* is not possible. Immunochemical techniques have been developed to address these issues.

Although, native DNA is not normally immunogenic,[10] modification by such ROS generating systems as methylene blue (MB) and white light, has been shown to increase the immunogenicity of DNA in experimental animals.[11] Furthermore, the MB/white light system has been shown to modify deoxyguanosine almost exclusively, leading to the formation of 8-oxodeoxyguanosine (8-oxodG) in DNA.[12] Immunisation with modified DNA has been one approach for the production of polyclonal antisera to specific oxidative DNA products, such as thymine glycol[13] and 8-oxoadenine.[14] An alternative approach has been adopted by some groups, whereby the immunogen comprised an oxidatively modified ribonucleoside, covalently attached to a protein. Both polyclonal[15] and monoclonal[16] antibodies have been produced to 8-oxodG by this method. However, whilst these antibodies have been reported to specifically recognise 8-oxodG in competitive ELISA assays, the successful demonstration of their binding to the damaged DNA duplex has largely been elusive.[15,16] Explanation, in part, may appear to have been provided by another group, producing antibodies by a phage display technique, who found that one of their clones to 8-oxodG, required the carrier protein to also be present and postulated that it was needed to stabilise antibody binding.[17]

We have used the modified ribonucleoside, 8-oxoguanosine, linked to protein to immunise both rabbits and mice in order to produce poly- and monoclonal antibodies to 8-oxodG, based on the methods of Degan *et al.*[15] and Park *et al.*[16] Screening of the rabbit polyclonal sera showed no discriminatory response between native DNA and DNA containing increased amounts of 8-oxodG (methylene blue-modified DNA – MB DNA). However, screening of mouse polyclonal sera and a resultant monoclonal antibody revealed good discrimination between native and MB DNA, although only when it had been rendered single-stranded. This lead us to develop a hypothesis relating antibody recognition to the conformation of 8-oxodG in DNA.

MATERIALS AND METHODS

Materials

Calf thymus DNA was from Calbiochem (Nottingham, UK) and only batches with an A_{260}/A_{280} ratio greater than or equal to 1.8 were used. The following reagents were obtained from Sigma (Sigma-Aldrich Chemical Company, Poole, UK): methylene blue, phosphate buffered saline (PBS, 0.01 M; pH 7.4; prepared from the tablet form); ascorbic acid; guanosine; sodium phosphate; keyhole limpet haemocyanin; methylated bovine serum albumin; sodium periodate; sodium carbonate; sodium borohydride and sulphuric acid. Fisher Scientific Ltd (Loughborough, UK) was the source of the ethanol (HPLC grade); methanol (HPLC grade). Charcoal powder and hydrochloric acid were from BDH laboratory supplies, Poole, UK. Ferric sulphate was obtained from Aldrich Chemical Company (Gillingham, UK).

Methods

Synthesis of 8-oxoguanosine (8-oxoGR)

The applicability of the periodate linkage method requires that the ribonucleoside derivative of the

base lesion be used. Therefore, 8-oxoguanosine (8-oxoGR), was synthesised by a method based on the Udenfriend system.[18] Guanosine (1 g) was dissolved in 1560 mL of 0.13 M sodium phosphate buffer (pH 6.8). One hundred and forty mL of 0.1 M ascorbic acid, 65 mL of 0.1 M EDTA and 13 mL of 0.1 M $FeSO_4$ were then added successively. Oxygen gas was then bubbled through the solution at 37°C for three hours, in the dark. The pH was then adjusted to 3.7 with 1 M HCl. Ten g of charcoal powder was then added with stirring. The mixture was then poured into a glass column with a sintered glass filter and allowed to settle. The column was drained and the residue washed with ultrapure water. The material adsorbed to the charcoal was then eluted with aqueous acetone (500 mL, 1 : 1 v/v) and the resulting eluent was then freeze-dried. The crude preparation was then dissolved in a minimum volume of HPLC grade methanol (10 mL).

Analytical HPLC of Crude 8-oxoguanosine Preparation

Analysis of the crude preparation was performed by a Gilson (Gilson Medival Electronics Ltd) system, consisting of a 306 pump, 806 Manometric module, 811c dynamic mixer, 712 HPLC controller, along with ECD and diode array detection (DAD). The mobile phase was 50 mM potassium phosphate and 10% methanol (v/v) at pH 5.5 used at a flow rate of 1 mL/min. Forty µL samples were injected onto a 25 cm Phase Sep ODS2, C-18 column (4.6 mm internal diameter). Following confirmation of a successful synthesis, preparative HPLC was performed to isolate the 8-oxoGR.

Preparative HPLC of Crude 8-oxoguanosine Preparation

This was performed on the system described above, with the exception that detection was solely performed by a Ranin Dynamax UV1 Absorbance detector. Typically 2500 µL injections were made onto a fully endcapped, 25 cm Phase Sep, Spherisorb ODS2 column (20 mm internal diameter). The mobile phase was 50 mM potassium phosphate and 10% (v/v) methanol at pH 5.5 at a flow rate of 8 mL/min. Four mL fractions were collected every half minute. Collected fractions of the peak eluting just after the main peak of guanosine were examined spectrophotometrically, to confirm the presence of 8-oxoguanosine. The collected samples were then pooled and re-run on the above system with 2.5% (v/v) methanol in ultrapure water as the mobile phase to purify the sample from the salts. A sample of the collected fraction was confirmed to be 8-oxoguanosine by electrospray mass-spectrometry.

Preparation of Nucleoside–Protein Conjugates

The 8-oxoGR was subsequently treated with periodate and linked either to keyhole limpet haemocyanin (KLH), or methylated bovine serum albumin (BSA), which were to act as protein carriers based on the method of Erlanger *et al.*[19] The conjugates were then extensively dialysed against 0.01 M PBS, pH 7.4, at 4°C. The solution was then freeze-dried and reconstituted in ultrapure water to 5 mg/mL protein. Guanosine was also linked to BSA by the above protocol and denoted GR/BSA. It is important to note that whilst the steps prior to linkage open the ribose ring, conjugation and stabilisation subsequently close the ring.

Antibody Production

Female, 6 week old mice were immunised with the 8-oxoguanosine/KLH conjugate (8-oxoGR/KLH) according to the following protocol. Reference bleeds were obtained prior to immunisation. The 8-oxoguanosine/KLH conjugate was then homogenised by whirlimixing 1 : 1 with Freund's Complete Adjuvant (Sigma). The mice ($n = 4$) were immunised subcutaneously with 50 µL immunogen mix per site, at multiple sites.

The animals were boosted subcutaneously with 50 μg immunogen in 100 μL sterile saline four weeks later. Test bleeds were performed 10 days post-boost. On achievement of a suitable response, the animals were sacrificed, three days after a final intra peritoneal boost (50 μg immunogen in 100 μL sterile saline), and spleen and sera collected. The latter was assessed by enzyme-linked immunosorbent assay (ELISA). Splenocytes were isolated from the spleen and hybridomas prepared with mouse myeloma cell line T80 NS0 (ECACC #85110503). Culture medium from HAT- (Hybri-max, Sigma) resistant hybridomas was screened by ELISA, in which neat culture medium and test bleed, whole serum acted as negative and positive controls, respectively. Initial screenings were performed with 8-oxoguanosine/BSA conjugate as the solid phase antigen. In later screenings this was then replaced by double- and single-stranded methylene blue-damaged DNA as this was deemed to possess a greater number of antigenic determinants. The hybridomas exhibiting a favourable response were cloned by limiting dilution.

Preparation of Methylene Blue Treated DNA (MB DNA)

The procedure is based on the method of Seaman *et al.*[111] and Floyd *et al.*[20] Briefly, calf thymus DNA (0.5 mg/mL in water, final concentration) was irradiated, with a white light source, in the presence of methylene blue (20 μg/mL, final concentration in 0.1 M Tris, pH 8.5) on ice, shielded from the light source by 0.5 cm of water. After irradiation, solid sodium chloride was added to the DNA solution to a final concentration of 1 M and the DNA precipitated, on ice, by the addition of ethanol until a precipitate formed. The DNA was then removed and dissolved in a minimum volume of deionised water. This was repeated twice to remove all traces of methylene blue. A DNA solution incubated with methylene blue but not exposed to light, and an untreated DNA solution acted as controls.

Determination of Levels of 8-oxo-2′deoxyguanosine in DNA

Enzymatic DNA hydrolysis Magnesium chloride (0.3 M in distilled water) was added to the DNA samples to give a final concentration of 10 mM. The solutions were then heated for 3 min at 100°C prior to rapid cooling on ice. The samples were then incubated at 37°C overnight in the presence of DNase I (0.1 mg/mg DNA) and endonuclease (0.14 units/μg DNA). Following adjustment of the pH to 8.0 with Tris base (1 M), phosphodiesterase (0.04 units/mg DNA) and alkaline phosphatase (1 unit/mg DNA) were added and the samples incubated overnight at 37°C. The pH was readjusted to 7.0 with 1 N HCl and analysed as described below.

Analytical HPLC of 8-oxodeoxyguanosine The amount of 8-oxodeoxyguanosine present in the enzymatically digested MB DNA was determined by reversed-phase HPLC using an EG&G model 400 electrochemical detector (Princeton Applied research, Princeton, USA) at +0.6 V working potential, versus an Ag/AgCl reference electrode. The mobile phase was 50 mM sodium acetate buffer pH 5.1, containing 1 mM ethylenediamine tetra-acetic acid and 5% (v/v) methanol, at a flow rate of 1 mL/min. Injections of 50 μL per sample were made onto a 3 μm ODS Hypersil column (100 mm × 4.6 mm i.d) from Shandon Scientific Ltd (Runcorn, UK). Deoxyguanosine was determined by ultraviolet detection (254 nm) with a model 168 diode array detector (DAD). This enabled the results to be expressed as moles of 8-oxodG per mole of dG.

Enzyme-linked Immunosorbent Assay

The ELISA solid phase antigens used consisted of either the 8-oxoGR/BSA conjugate or methylene blue DNA, the production of which is described above. The solid phase antigen, (50 μg/mL protein or 50 μg/mL DNA), 50 μL/well, was bound to a 96 well Nunc, Immuno Maxisorp ELISA plate (Life Technologies Ltd, Paisley, Scotland) by incubation, in a humidified environment at 37°C

for 1 h, after which the plate was washed three times with PBS. Free sites were then blocked by incubation with 150 μL/well 4% (w/v) dried skimmed milk (Tesco Stores Ltd, Cheshunt, UK) in PBS (4% milk/PBS (w/v)) for 1 h at 37°C in a humidified environment and the wells were then washed with PBS. The test antiserum, 50 μL/well, was used at a dilution of 1/10, 1/100 and 1/1000 in 4% milk/PBS (w/v) and incubated for 1 h as described above. Following washing three times with PBS containing 0.05% (v/v) Tween 20 (Sigma), the secondary antibody, a peroxidase-labelled goat anti-rabbit immunoglobulins (IgA, IgM and IgG – DAKO Ltd, High Wycombe, UK) at a 1/2000 dilution in milk PBS, was applied (50 μL/well). After the plate had been incubated as previously described, and washed with PBS/Tween 20, the substrate solution, orthophenylenediamine (0.5 mg/mL in 0.05 M phosphate–citrate, pH 5.0 and containing 0.03% w/v sodium perborate) was added (50 μL/well) and incubated for 15 min at room temperature. The reaction was stopped using 25 μL/well 2 M H_2SO_4. The resulting absorbance was read at 492 nm, using an Anthos 2001 plate reader (Anthos Labtec Instr.). The final data included correction for background values.

Statistical analyses were performed using Graph Pad Prism, version two.

RESULTS

Preparation of 8-oxoguanosine

As stated earlier, the deoxynucleoside form of the lesion is unsuitable for periodate linkage to protein. However, Mueller and Rajewsky,[21] on describing their periodate-linked immunogen, O^6-ethylguanosine, state that such a conjugate structurally resembles more closely the deoxyribonucleoside than the ribonucleoside, making such a linkage highly appropriate for our lesion. Therefore, in order to raise antibodies to 8-oxodG, 8-oxoGR was first synthesised, positively identified and then conjugated to a carrier protein prior to immunisation.

HPLC-ECD analysis of the reaction products from the Udenfriend system revealed an electrochemically active peak, with a retention time of 9.5 min (data not shown), which suggested that 8-oxoGR had been produced. Collected fractions, corresponding to the peak of interest, were collected by preparative HPLC and examined spectrophotometrically for the characteristic 8-oxoGR spectrum by comparison with a standard. Following desalting, the samples were pooled and electrospray mass spectrometry (EMS) was performed, the results of which indicated the peak of interest at M_w 322 to be hydroxylated guanosine which had gained both H^+ and Na^+, as part of the EMS procedure (Figure 1).

Immunogenicity of 8-oxoGR in Rabbits

Following booster injections with 8-oxoGR/KLH an immunogenic response was noted over pre-immune, which improved over the first three to four months, achieving a maximum titre of 1/548 and 1/450 against MB DNA, in the two experimental animals respectively. Treatment of DNA with methylene blue, in conjunction with white light, has been shown to produce the almost exclusive induction of 8-oxodG.[12] HPLC analysis of the MB DNA showed levels of one 8-oxodG per 100 dG (data not shown) and therefore significantly modified compared to native.[22] Double-stranded MB DNA was therefore used as the solid phase antigen as it was likely to possess more antigenic sites than the prepared 8-oxoguanosine/BSA conjugate (8-oxoBSA). It was also noted that the response of these antisera against MB DNA was strikingly similar to the response against native DNA, a facet seen in both animals.

It appeared that a maximum titre, in both animals, had been achieved during this immunisation schedule, although subsequent immunisations, instead of increasing the titre further,

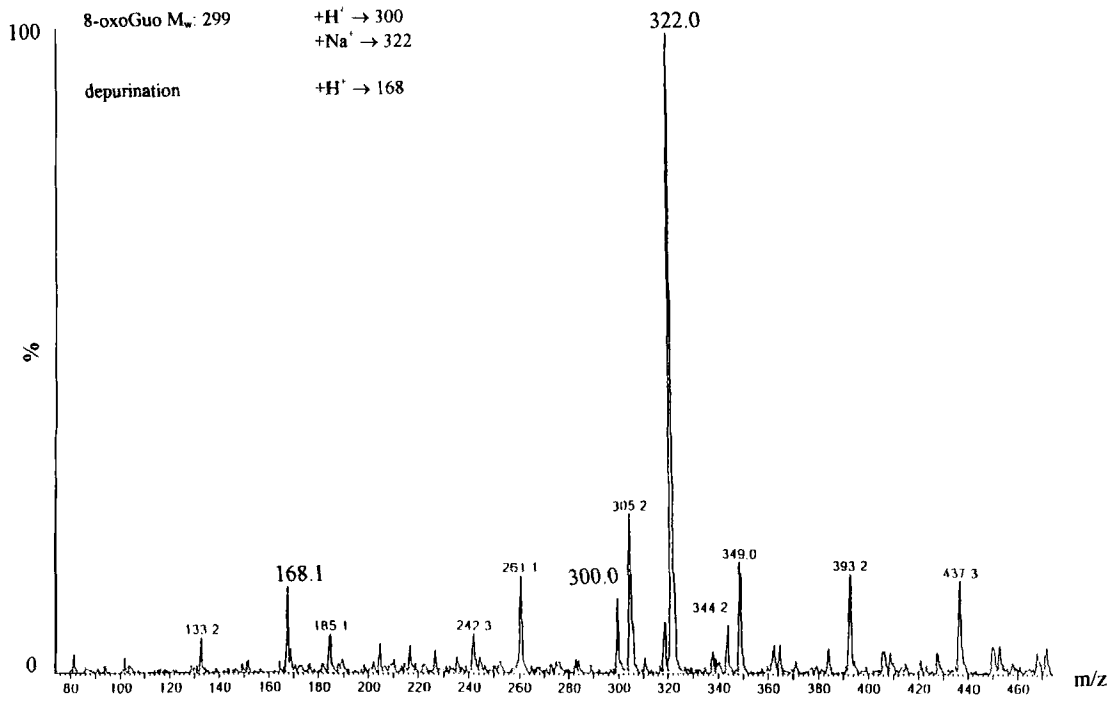

FIGURE 1 Representative electrospray mass spectrum of the purified, collected fraction. The peak of interest is M_w 322, demonstrating that the fraction contains hydroxylated guanosine (8-oxoGR).

merely resulted in a loss of response. Nonetheless, a response to immunisation with DNA treated with methylene blue and white light had been achieved and thus warranted progression to a monoclonal system in order to attempt to select clone(s) producing the antibodies which could differentiate between native and MB DNA.

Screening of Mouse Antibodies

Following booster injections with 8-oxoGR/KLH an immune response was produced in all four mice, against double-stranded MB DNA, compared to pre-immune. Representative ELISA absorbances from a 1:1000 dilution of serum are 0.1 for pre-immune, versus 1.25, post-boost. Again no discrimination was demonstrated between modified and native double-stranded DNA (Figure 2A). However, further examination of the polyclonal response demonstrated that rendering both the native and MB DNA single-stranded produced a greater than two-fold discrimination of MB DNA over native, at all dilutions of the antiserum (Figure 2B).

Initial screening of hybridomas revealed one culture (denoted 3/8) to be producing a response over the medium control. This was subsequently cloned, producing three secreting clones (denoted 3/8/1, 3/8/2 and 3/8/3). Clone 3/8/1 appeared to be producing the best response to 8-oxoBSA compared to the medium control (data not shown). This clone was termed a monoclonal as a result of the serial dilution process adopted during its culturing. Further screening of this clone showed a failure to discriminate between double-stranded MB DNA and native (Figure 3), as had been seen with the polyclonal sera. However, denaturation of the double-stranded MB DNA resulted in a two-fold increase in binding of 3/8/1 immunoglobulin (Figure 3), determined as being significant ($p < 0.01$).

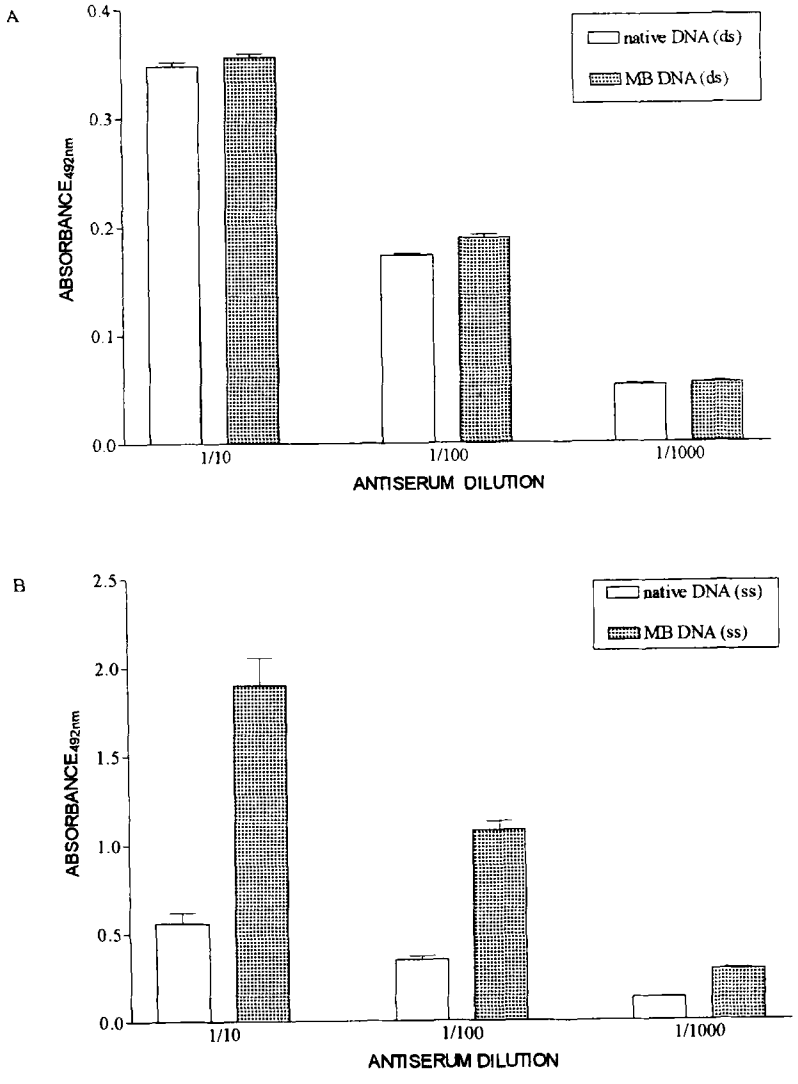

FIGURE 2 A. Representative direct ELISA binding of antiserum from a single mouse (#3) immunised with 8-oxoguanosine conjugated to keyhole limpet haemocyanin, to double-stranded (ds) native (nDNA) and methylene blue-treated DNA (MB DNA). Values represent the mean (SEM) of triplicate determinations per dilution. B. Representative direct ELISA binding of antiserum from a single mouse (#3) immunised with 8-oxoguanosine conjugated to keyhole limpet haemocyanin, to single-stranded (ss) native (nDNA) and methylene blue-treated DNA (MB DNA). Values represent the mean (SEM) of triplicate determinations per dilution.

Additionally, use of the 8-oxoGR/BSA conjugate also resulted in an increase in binding over a guanosine/BSA conjugate (GR/BSA). Binding to native DNA, irrespective of whether it was denatured or not, was two-fold lower than to single-stranded MB DNA.

These data, although not fully characterising the antibody, would suggest that recognition of the lesion in DNA depends on the hydroxylation at the guanine C-8 position in conjunction with denaturation of the duplex.

DISCUSSION

We have generated antibodies with the ability to discriminate between native and oxidatively

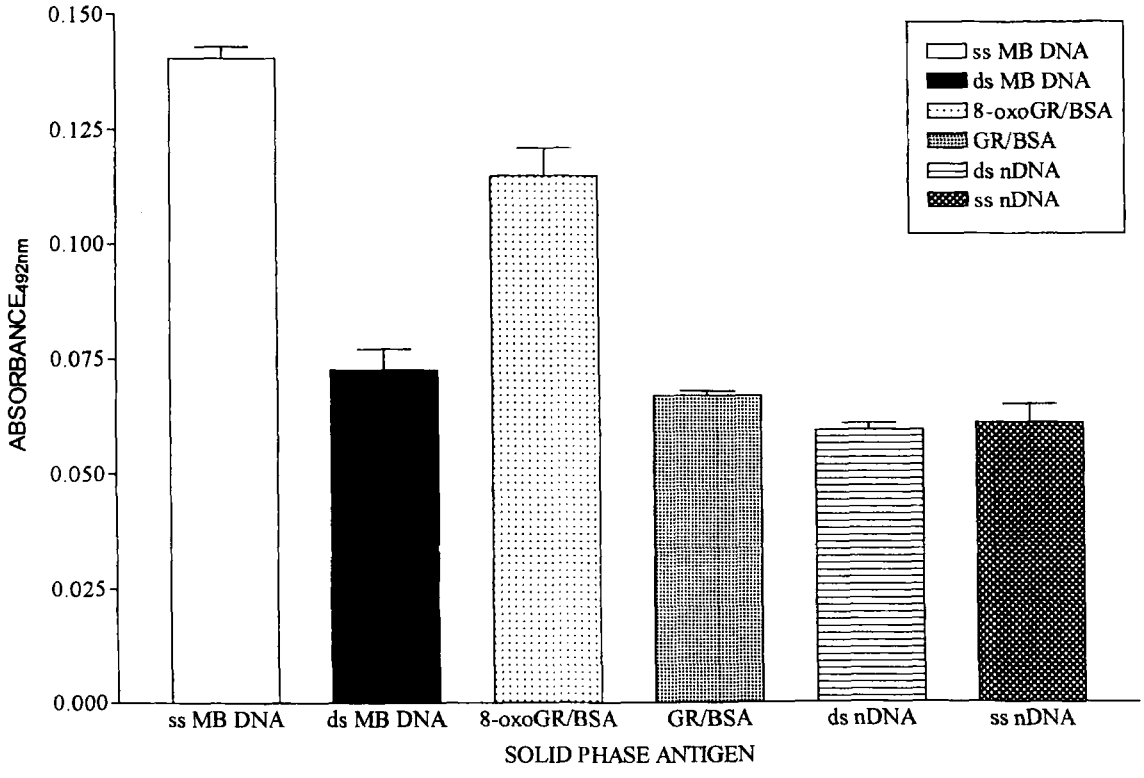

FIGURE 3 Direct ELISA binding of immunoglobulin in supernatant from clone 3/8/1 to single-stranded (ss) and double-stranded (ds) methylene blue modified (MB DNA), native DNA (nDNA), the guanosine/BSA conjugate (GR/BSA) and the 8-oxoguanosine/BSA conjugate (8-oxoGR/BSA). Values represent the mean (SEM) of triplicate determinations.

modified DNA. Furthermore, these antibodies have been shown to be present both in whole mouse antiserum and in the supernatant of a hybridoma cell line. From this evidence we propose a hypothesis in which glycosidic conformation of 8-oxodG is a factor influencing both the immunogenicity and antigenicity of this lesion.

The approach to developing antibodies which recognise oxidative damage has broadly followed two routes; (i) the development of polyclonal antisera to DNA modified with a multiplicity of lesion types, or (ii) monoclonal antibody production to specific lesions. The literature reports numerous methods for the induction of ROS damage to DNA (ROS DNA), including iron (II) sulphate,[23] ascorbate[24] or UVC,[25] all in conjunction with hydrogen peroxide. Furthermore, all have been utilised in an attempt to produce polyclonal antisera to ROS-induced DNA damage.[26] The lesion of choice for monoclonal production has been 8-oxodG, proposed to be a biomarker of oxidative DNA damage. This approach for monoclonals, first successfully reported by Park *et al.*,[16] necessitated the synthesis of the modified guanine ribonucleoside and conjugation with a carrier protein prior to immunisation.

Using 8-oxoguanosine linked to bovine serum albumin as an immunogen, we have generated both rabbit and mouse polyclonal responses, along with a monoclonal response, all of which failed to discriminate between double-stranded native and MB DNA. The latter DNA treatment was shown to be extensively modified, containing elevated levels of 8-oxodG and thus represents a good system in which to screen an experimental antiserum. Such a failure to

discriminate would perhaps not be surprising as the modified deoxynucleoside is structurally different from the native form only by a carbonyl group at the C-8 position of the purine ring – resulting in antisera which are raised to 8-oxoGR recognising dG. Some cross-reactivity with the native base and deoxynucleoside has also been noted in the competition RIAs of Park *et al.*[16] and, to a lesser extent, Degan *et al.*[15] with their mono- and polyclonal antibodies, respectively. As a result the authors dismiss their usefulness in quantitating endogenous levels of 8-oxodG in intact DNA.

However, we have noted that upon denaturation of native and MB DNA, a two-fold discrimination can be elicited using either the mouse poly- or monoclonal antibodies. This increase in recognition is believed to be, in part, due to conformational changes of the 8-oxodG in single-stranded DNA. In DNA, guanine normally adopts the keto form with an *anti* glycosidic conformation, resulting in base-pairing with cytosine. On formation in double-stranded DNA, 8-oxodG, of which the 6,8-diketo form predominates under physiological conditions,[27] is thought to be held in the *anti* form, adopting a normal Watson–Crick base pair with dC. The presence of the modified base does not lead to any structural distortion of the duplex, but NMR and UV melting techniques reveal a slight duplex destabilisation.[28] This probably arises from steric repulsion between the oxygen atom at the C-8 position and the ribofuranose ring of the same.[29] It is for this reason that 8-oxodG is prone to adopting the energetically favourable *syn* glycosidic conformation which possesses the ability to distort the DNA making normal base-pairing unsuitable.[30] Whilst pairing with dC may still occur, the *syn* conformation of 8-oxodG makes mis-pairing with dA and T possible.[31] However, any resultant structural changes to the DNA are subsequently located near the site of modification.[28]

Taken together, these data suggest that monomeric 8-oxoGR/KLH in the immunogen resembles the *syn* glycosidic conformation of 8-oxodG and as a consequence it is this with which the immune system is presented. Furthermore, the development of antibodies to this structure implicate its orientation in immunogenicity. Antibodies have previously demonstrated a potential to detect conformational changes related to DNA lesions.[32] Furthermore, Sanford *et al.*[32] noted that the glycosidic conformation of the guanine residues involved in the binding sites of two anti-Z-DNA antibodies was in fact *syn*, not the native *anti*. It is therefore possible that in the system used here, antibodies specific to *syn* 8-oxodG may have been generated. Within double-stranded DNA the lesion is unlikely to possess such a conformation due to it being held in place (in the *anti* conformation) by base-pairing with cytosine. Larger conformational changes of the DNA duplex may be introduced by the torsion exerted as the 8-oxodG attempts to swap to the energetically favourable *syn* conformation – leading to limited exposure of the lesion and some recognition by the antisera, as seen in our results. However, rendering the DNA single-stranded allows the 8-oxodG to adopt the *syn* conformation as resembled in the immunogen. It would therefore appear that the combination of the *anti* to *syn* conformational change, along with the modification at the C-8 position, produces recognition of 8-oxodG over dG in single-stranded DNA. Degan *et al.*[15] also speculated that conformational changes may play a role in the difference in binding affinities for 8-oxodG and dG of their antibodies, although rendering damaged DNA single-stranded did not enhance discrimination with their antibodies, making useful application in DNA impractical. Indeed, Ide *et al.*[33] considered conformational changes to be reponsible for the differences in reactivity of their antibodies to 8-oxoadenosine and 8-oxoGR when in free solution or DNA.

Bespalov *et al.*[17] hypothesised that their recombinant Phabs may not recognise 8-oxodG in DNA due to the requirement of bovine serum albumin, the immunogen carrier, to stabilise

binding. We suggest that the difference in lesion conformation between the immunogen resembling *syn* 8-oxoguanosine and *anti* 8-oxodG in double-stranded DNA, may also be an important factor. Furthermore, both the polyclonal antiserum of Degan *et al.*[15] and monoclonal antibodies of Park *et al.*[16] failed to recognise 8-oxodG in DNA, whilst successfully recognising the free deoxynucleoside in competitive ELISA. This would suggest that whilst stabilisation of binding by a carrier protein is not vital, some other component is. We therefore propose the glycosidic conformation of 8-oxodG may be an important factor in the recognition of 8-oxodG as an antigenic determinant and that denaturation of the DNA duplex may enhance the discrimination of some previously reported antibodies to 8-oxodG.

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References

[1] Bunker, V.W. (1992) Free radicals, antioxidants and ageing. *Medical Laboratory Science*, **49**, 299–312.

[2] Frenkel, K., Karkoszka, J., Kim, E. and Taioli, E. (1993) Recognition of oxidised DNA bases by sera of patients with inflammatory diseases. *Free Radical Biology and Medicine*, **14**, 483–494.

[3] Lunec, J., Herbert, K., Blount, S., Griffiths, H.R. and Emery, P. (1994) 8-hydroxydeoxyguanosine: a marker of oxidative DNA damage in systemic lupus erythematosus. *Federation of European Biochemical Societies Letters*, **348**, 131–138.

[4] Floyd, R.A. (1990) The role of 8-hydroxyguanine in carcinogenesis. *Carcinogenesis*, **11**, 1447–1450.

[5] Ward, J.F., Evans, J.W., Limoli, C.L. and Calabro-Jones, P.M. (1987) Radiation and hydrogen peroxide induced free radical damage to DNA. *British Journal of Cancer*, **55**, 105–112.

[6] Halliwell, B. (1993) Oxidative DNA damage: meaning and measurement. In *DNA and Free Radicals* (eds. B. Halliwell and O. I. Aruoma), Ellis Horwood, New York, pp. 67–79.

[7] Floyd, R.A., Watson, J.J., Wong, P.K., Altmiller, D.H. and Rickard, R.C. (1986) Hydroxyl free radical adduct of deoxyguanosine: sensitive detection and mechanism of formation. *Free Radical Research Communications*, **1**(3), 163–172.

[8] Dizdaroglu, M. (1991) Chemical determination of free radical-induced damage to DNA. *Free Radical Biology and Medicine*, **10**, 225–242.

[9] Ravanat, J.-L., Turesky, R.J., Gremaud, E., Trudel, L.J. and Stadler, R.H. (1995) Determination of 8-oxoguanine in DNA by gas chromatography–mass spectrometry and HPLC-electrochemical detection: overestimation of the background level of oxidised base by the gas chromatography–mass spectrometry assay. *Chemical Research in Toxicology*, **8**, 1039–1045.

[10] Plescia, O.J., Braun, W. and Palczuk, N.C. (1964) Production of antibodies to denatured deoxynucleic acid (DNA). *Biochemistry*, **52**, 279–285.

[11] Seaman, E., Levine, L. and Van Vanakis, H. (1966) Antibodies to methylene blue sensitised photooxidation products in deoxyribonucleic acid. *Biochemistry*, **5**, 1216–1223.

[12] Epe, B. (1991) Genotoxicity of singlet oxygen. *Chemico-Biological Interactions*, **80**, 239–260.

[13] West, G.J., West, I.W.-L. and Ward, J.F. (1982) Radioimmunoassay of a thymine glycol. *Radiation Research*, **90**, 595–608.

[14] West, G.J., West, I.W.-L. and Ward, J.F. (1982) Radioimmunoassay of 7,8-dihydro-8-oxoadenine (8-hydroxyadenine). *International Journal of Radiation Biology*, **42**, 481–490.

[15] Degan, P., Shigenaga, M.K., Park, E.M., Alperin, P.E. and Ames, B.N. (1991) Immunoaffinity isolation of urinary 8-hydroxy-2′-deoxyguanosine and 8-hydroxyguanine and quantitation of 8-hydroxy-2′-deoxyguanosine in DNA by polyclonal antibodies. *Carcinogenesis*, **12**, 865–871.

[16] Park, E.M., Shigenaga, M.K., Degan, P., Korn, T.S., Kitzler, J.W., Weher, C.M., Kolachana, P. and Ames, B.N. (1992) Assay of excised oxidative DNA lesions: Isolation of 8-oxoguanine and its nucleoside derivatives from biological fluids with a monoclonal antibody column. *Proceedings of the National Academy of Sciences USA*, **89**, 3375–3379.

[17] Bespalov, I.A., Purmal, A.A., Glackin, M.P., Wallace, S.S. and Melamede, R.J. (1996) Recombinant Phabs reactive with 7,8-dihydro-8-oxoguanine, a major oxidative DNA lesion. *Biochemistry*, **35**, 2067–2078.

[18] Kasai, H., Nishimura, S. (1984) Hydroxylation of deoxyguanosine at the C-8 position by ascorbic acid and other reducing agents. *Nucleic Acids Research*, **12**, 2137–2145.

[19] Erlanger, B.F. and Beiser, S.M. (1964) Antibodies specific for ribonucleosides and ribonucleotides and their reaction with DNA. *Proceedings of the National Academy of Sciences USA*, **52**, 68–74.

[20] Floyd, R.A., West, M.S., Eneff, K.L. and Schneider, J.E. (1989) Methylene blue plus white light mediates 8-hydroxyguanine formation in DNA. *Archives of Biochemistry and Biophysics*, **273**, 106–111.

[21] Mueller, R. and Rajewsky, M.F. (1980) Immunological quantification by high-affinity antibodies of O^6-ethyldeoxyguanosine in DNA exposed to N-ethyl-N-nitrosourea. *Cancer Research*, **40**, 887–896.

[22] Schneider, J.E., Price, S., Maidt, L., Gutteridge, J.M.C. and Floyd, R.A. (1991) Methylene blue plus light mediates 8-hydroxy 2′-deoxyguanosine formation in DNA preferentially over strand breakage. *Nucleic Acids Research*, **18**, 631–635.

[23] Alam, K., Ali, A. and Ali, R. (1993) The effect of the hydroxyl radical on the antigenicity of native DNA. *Federation of European Biochemical Societies*, **319**, 66–70.

[24] Blount, S., Griffiths, H., Emery, P. and Lunec, J. (1990) Reactive oxygen species modify human DNA, eliciting a more discriminating antigen for the diagnosis of systemic lupus erythematosus. *Clinical Experimental Immunology*, **81**, 384–389.

[25] Ara, J. and Ali, R. (1993) Polynucleotide specificity of anti-reactive oxygen species (ROS) DNA antibodies. *Clinical Experimental Immunology*, **94**, 134–139.

[26] Cooke, M.S., Mistry, N., Wood, C., Herbert, K.E. and Lunec, J. (1997) Immunogenicity of DNA damaged by reactive oxygen species – implications for anti-DNA antibodies in Lupus, *Free Radical Biology and Medicine*, **22**, 151–159.

[27] Aida, M. and Nishimura, S. (1987) An *ab initio* molecular orbital study on the characteristics of 8-hydroxyguanine. *Mutation Research*, **192**, 83–89.

[28] Oda, Y., Uesugi, S., Ikehara, M., Nishimura, S., Kawase, Y., Ishikawa, H., Inoue, H. and Ohtsuka, E. (1991) NMR studies of a DNA containing 8-hydroxydeoxyguanosine. *Nucleic Acids Research*, **19**, 1407–1412.

[29] Brown, T. and Kennard, O. (1992) Structural basis of DNA mutagenesis. *Current Opinion in Structural Biology*, **2**, 354–360.

[30] Culp, S.J., Bongsup, P.C., Kadlubar, F.F. and Evans, F.E. (1989) Structural and conformational analyses of 8-hydroxy-2′-deoxyguanosine. *Chemical Research in Toxicology*, **2**, 416–422.

[31] Lipscomb, L.A., Peek, M.E., Morningstar, M.L., Verghis, S.M., Miller, E.M., Rich, A., Essigmann, J.M. and Williams, L.D. (1995) X-ray structure or a DNA decamer containing 7,8-dihydro-8-oxoguanine. *Proceedings of the National Academy of Sciences USA*, **92**, 719–723.

[32] Sanford, D.G. and Stollar, B.D. (1990) Characterisation of anti-Z-DNA antibody binding sites on Z-DNA by nuclear magnetic resonance spectroscopy. *Journal of Biological Chemistry*, **265**, 18608–18614.

[33] Ide, H., Kow, Y.W., Chen, B.-X., Erlanger, B.F. and Wallace, S.S. (1997) Antibodies to oxidative DNA damages: Characterisation of antibodies to 8-oxopurines. *Cell Biology and Toxicology*, **13**, 405–417.