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Monoclonal Antibody to Single-Stranded DNA: A Potential Tool for DNA Repair Studies

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Growing evidence suggests that DNA repair capacity is an important factor in cancer risk and is therefore essential to assess. Immunochemical assays are amenable to the detection of repair products in complex matrices, such as urine, facilitating noninvasive measurements, although diet and extra-DNA sources of lesion can confound interpretation. The production of single-stranded, lesion-containing DNA oligomers characterises nucleotide excision repair (NER) and hence defines the repair pathway from which a lesion may be derived. Herein we describe the characterisation of a monoclonal antibody which recognises guanine moieties in single-stranded DNA. Application of this antibody in ELISA, demonstrated such oligomers in supernatants from repair-proficient cells post-insult. Testing of urine samples from volunteers demonstrated a relationship between oligomer levels and two urinary DNA damage products, thymine dimers and 8-oxo-2′-deoxyguanosine, supporting our hypothesis that NER gives rise to lesion-containing oligomers which are specific targets for the investigation of DNA repair.

Key Words: **antibodies; DNA damage; repair; thymine dimers, 8-oxo-2′-deoxyguanosine, xeroderma pigmentosum; urine.**

DNA damage, repair and mutation are reported to be important events in carcinogenesis (1). Assessing these processes is therefore a vital approach to elucidating the pathogenesis of cancer, identifying potential intervention strategies, measuring exposure and determining disease risk (2). Numerous approaches have been reported to investigate these issues, including the comet assay (3), GC-MS (4), LC-MS/MS (5), HPLC (6) and ^{32}P-postlabelling (7). Immunochemical assays in particular, have proven to be useful, largely due to their versatility in detecting lesions in a number of biological matrices, invariably without the need for time-consuming sample workup, often associated with chromatographic techniques (8). Antibodies specific to a wide variety of lesions have been used to locate or quantify lesions in cells (9), tissues (10), serum (11), cell culture supernatants (12) and urine (13). Indeed, it seems that apart from absolute quantitation, the sole difficulty with immunochemical methods derives from generating a suitable antibody (discussed in (14)).

The hypothesis concerning the potentially important role played by DNA repair in cancer risk is gaining experimental support (15). This is exemplified by the varying degrees of cancer susceptibility exhibited by xeroderma pigmentosum and related conditions, accounted for by defects in the nucleotide excision repair (NER) process (16). Furthermore, a number of polymorphisms have been identified in genes encoding for proteins which maintain the stability of the genome (17, 18), which may affect their effectiveness. Nucleotide excision repair is characterised by the removal of, largely bulky, adducts in the form of a single-stranded oligomer, approximately 24–29 bases long (19). The post-excision processing of these lesion-containing fragments is unclear, although it has been proposed that exonucleolytic degradation of the oligomer would occur until the lesion was reached leaving a lesion-containing 6- to 7-mer (20), which would ultimately appear in the urine.

There already exists methods for quantifying an individual's repair capacity, however these methods are involved, time consuming and invasive (21, 22)—requiring a blood sample. Immunoassays represent a simple means by which large-scale, high-throughput assessments might be made and are most suitable for analysing complex biological matrices, such as urine. However, current methods for measuring urinary DNA adducts, in particular oxidative lesions, can be confounded by diet and extra-DNA sources of the adduct and are hence not reflective of repair (23). We propose

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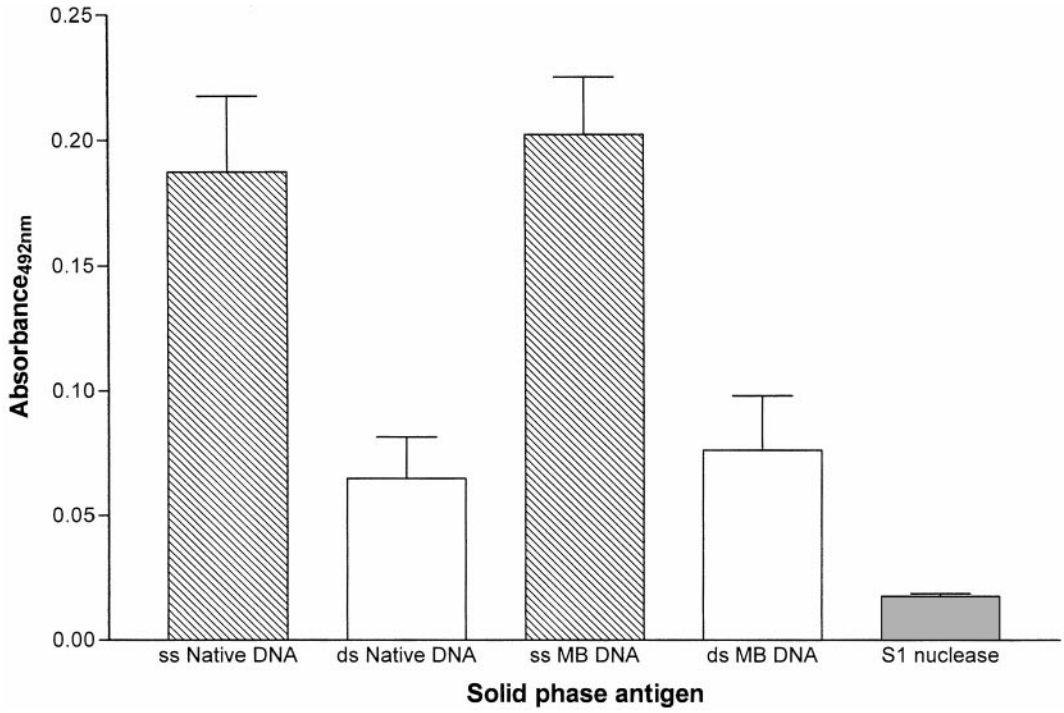

FIG. 1. Direct ELISA binding of undiluted supernatant from hybridoma F9/3-3 against single- and double-stranded (ss and ds, respectively) native and methylene blue DNA (MB DNA). Binding of F9/3-3 to S1 nuclease-treated single-stranded DNA (denoted S1 nuclease) is also shown. Error bars indicate the standard deviation of 13 determinations.

that it is possible to circumvent these issues by utilising an assay which would specify the repair pathway studied.

Herein we report the serendipitous finding of an antibody raised to the oxidative lesion 8-oxoguanosine, which recognises guanine moieties contained within single-stranded DNA. We speculate upon the significance of this finding within the context of autoimmune disease and report the potential use of this antibody in an *in vivo* repair assay.

MATERIALS AND METHODS

Ninety-six-well Nunc, Immuno Maxisorp ELISA plates were from Life Technologies Ltd. (Paisley, Scotland). Dried skimmed milk, was provided by Tesco Stores Ltd. (Cheshunt, UK). The secondary antibody, peroxidase-labelled goat anti-mouse immunoglobulins M was from DAKO Ltd. (High Wycombe, UK). Calf thymus DNA was from Calbiochem (Nottingham, UK). The following reagents were obtained from Sigma-Aldrich Chemical Company (Poole, UK): Tween 20, phosphate buffered saline (PBS, 0.01 M, pH 7.4; prepared from the tablet form), sodium phosphate, 2′-deoxyguanosine, 8-oxo-2′-deoxyguanosine (8-oxodG), and sulphuric acid.

Preparation of 8-oxoguanosine and methylene blue DNA. The ribonucleoside immunogen, 8-oxoguanosine, was synthesised by a method based on that of the Udenfriend system (24), as reported previously (25). Methylene blue DNA (MB DNA) was prepared using the procedure of Seaman *et al.* (26) and Floyd *et al.* (27). Analysis of the 8-oxodG content of the MB-DNA was performed by high performance liquid chromatography with electrochemical detection (HPLC-EC), following enzymatic digestion, as described by Cooke *et al.* (25).

Immunisation protocol for monoclonal antibodies to 8-oxoguanosine. Reference bleeds were obtained from six-week-old, female mice ($n = 4$) prior to immunisation. The 8-oxoguanosine/KLH conjugate was then homogenised 1:1 with Freund's Complete Adjuvant. The mice were immunised with 50 μL immunogen mix subcutaneously at multiple sites then boosted subcutaneously with 50 μg immunogen in 100 μL sterile saline four weeks later. Test bleeds were performed 10 days post-boost. The animals were sacrificed, three days after a final intraperitoneal boost, and spleen and sera collected when the appropriate titre of antibody had been obtained. The latter was assessed by enzyme-linked immunosorbant assay (ELISA). The spleen was collected into a Bijou bottle containing 5 mL of medium.

Enzyme-linked immunosorbant assay. The direct ELISA was performed using the appropriate solid phase antigen, as described previously (28).

Mouse monoclonal production. Production of mouse monoclonal antibodies and dilution cloning were performed as described previously (25). Screening was performed by competitive ELISA, once a favourable response was identified the clone was expanded and the antibody purified and characterised.

Antibody purification and characterisation. Cell culture supernatant from promising clones was purified by E-Z-Sep (Pharmacia Biotech.), according to the manufacturers instructions prior to characterisation by direct and competitive ELISA. Specificity for single-stranded DNA was determined by S1 nuclease treatment, as described previously (29).

Competitive ELISA. Competitive ELISA was performed for antibody characterisation. The method was essentially as previously described (28). The test supernatant/purified antibody was mixed with decreasing amounts of various competitors and then incubated in the wells for 1 h at 37°C. Detection of bound antibody was achieved as described for the noncompetitive ELISA.

Cell culture and treatment. SV40-transformed, control, disease-free lymphoblastoid cells (GM00131B from NIGMS Human Genetic Mutant Cell Repository) and a corresponding cell line derived from a xeroderma pigmentosum complementation group A patient (GM02250F from NIGMS Human Genetic Mutant Cell Repository) were maintained in 25 cm^3 tissue culture flasks in MEM Eagle Earle medium (pH 7.4), supplemented with 15% foetal bovine serum, 2× essential amino acids, 2× nonessential amino acids, and 2× vitamins at 37°C in a humidified atmosphere of 5% CO_2. Conditioned media was replaced every 2 days. The fibroblasts were subcultured (ratio 1:3) by removing conditioned media and washing the cells with phosphate buffered saline (PBS) pH 7.4. Treatment of both cells types was with 300 μM H_2O_2. Samples of culture supernatants were taken at 0, 1.3, 3.3, 7.3, 28.0, 30.3 and 40.3 h timepoints and assessed for oligomers, as described above, or T<>T, or 8-oxodG, by competitive ELISA, based upon the method of Ahmad *et al.* (30).

Urine samples. Urine samples were obtained from eleven healthy subjects (6 female, 5 male; median age 36, age range 20–56 years). Urinary 8-oxodG, T<>T and creatinine levels were determined as previously described (30). Creatinine levels were assessed, as impaired renal function may affect lesion levels excreted in the urine. Collected urine samples were stored at −80°C in 20 mL Universal tubes until analysis. Following thawing and centrifugation (300*g* for 10 min), the supernatants were assessed for oligomers, 8-oxodG (11) and T<>T (30) by competitive ELISA.

RESULTS

Antibody Characterisation

Initial screening of hybridomas for the secretion of an antibody to 8-oxodG resulted in only one positive, denoted F9/3 which was cloned. Screening of the cloned hybridoma revealed many clones showing positive reactivity to single-stranded MB-DNA, of which three were growing well (F9/3-3, -30 and -52) and expanded into larger culture flasks for supernatant collection. Isotyping of the clones F9/3-3, -30 and -52 revealed the antibodies to be IgM type. The most promising monoclonal hybridoma of the three, F9/3-3, was affinity purified and characterised. Direct ELISA assessment of the reactivity of F9/3-3 against native versus MB-DNA revealed a strong affinity for single-stranded DNA irrespective of whether the DNA contained significant levels of 8-oxodG (Fig. 1). The possible detection of double-stranded DNA was ruled out by treatment of double- and single-stranded DNA with S1 nuclease, an enzyme which digests only single-stranded DNA. Treatment of single-stranded DNA with S1 nuclease significantly reduced the binding of F9/3-3, approximately tenfold, proving a specificity for single-stranded DNA (Fig. 1).

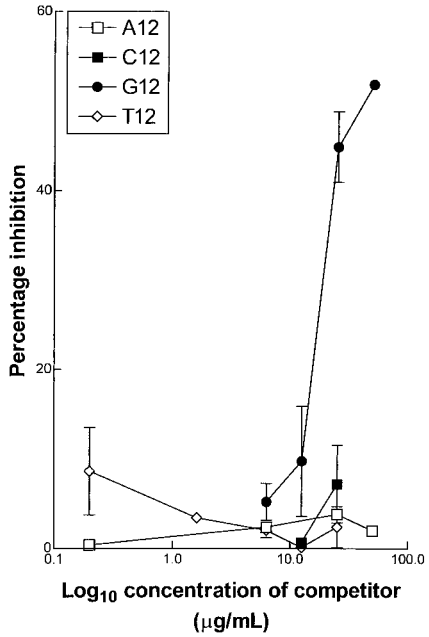

FIG. 2. Inhibition of purified antibody F9/3-3 binding to single-stranded native DNA by adenine-, cytosine-, guanine-, or thymine containing 12-mers (A_{12}, C_{12}, G_{12} and T_{12}, respectively). Maximum inhibition (100%) was that observed in the absence of solid phase antigen. Zero percent inhibition was I the absence of competitors. Values represent the mean and range of two determinations per concentration.

TABLE 1

Putative urinary constituent	IC_{50} (μg/mL)
Thymine	>500
Adenine	>500
Guanine	>500
Cytosine	>500
Bovine serum albumin	>250
8-oxoguanine	>250
8-oxo-2′-deoxyguanosine	>250
Deoxyguanosine	>250
Guanosine	>500
Creatinine	>500
Uric acid	>500
Urea	>500
Xanthine	>500

Competitive ELISA using polynucleotides further suggested deoxyguanosine specifically, to be involved in the antigenic determinant (Fig. 2), although not in the isolated form of the native, or oxidatively modified, base, ribonucleoside, or deoxyribonucleoside (Table 1). The specificity of F9/3-3 was investigated by using a series of oligomers containing increasing amounts of dG (G_2-G_{12}, Fig. 3). G_2 was the least effective oligomer to induce binding of F9/3-3, although recognition clearly occurred with a maximal absorbance of 0.25 units at a concentration of 100 μg/mL, indicating the minimum size for the antigenic determinant. Binding of F9/3-3 increased with increasing length of oligomer, with a maximal absorbance of 0.7 units for G_{12} at a concentration of approximately 10 μg/mL (Fig. 3).

In Vitro Assessment of Nucleotide Excision Repair

The appearance of repair-derived oligomers in cell culture medium was investigated using NER proficient and deficient (XPA (31)) cell lines. Given that T<>T

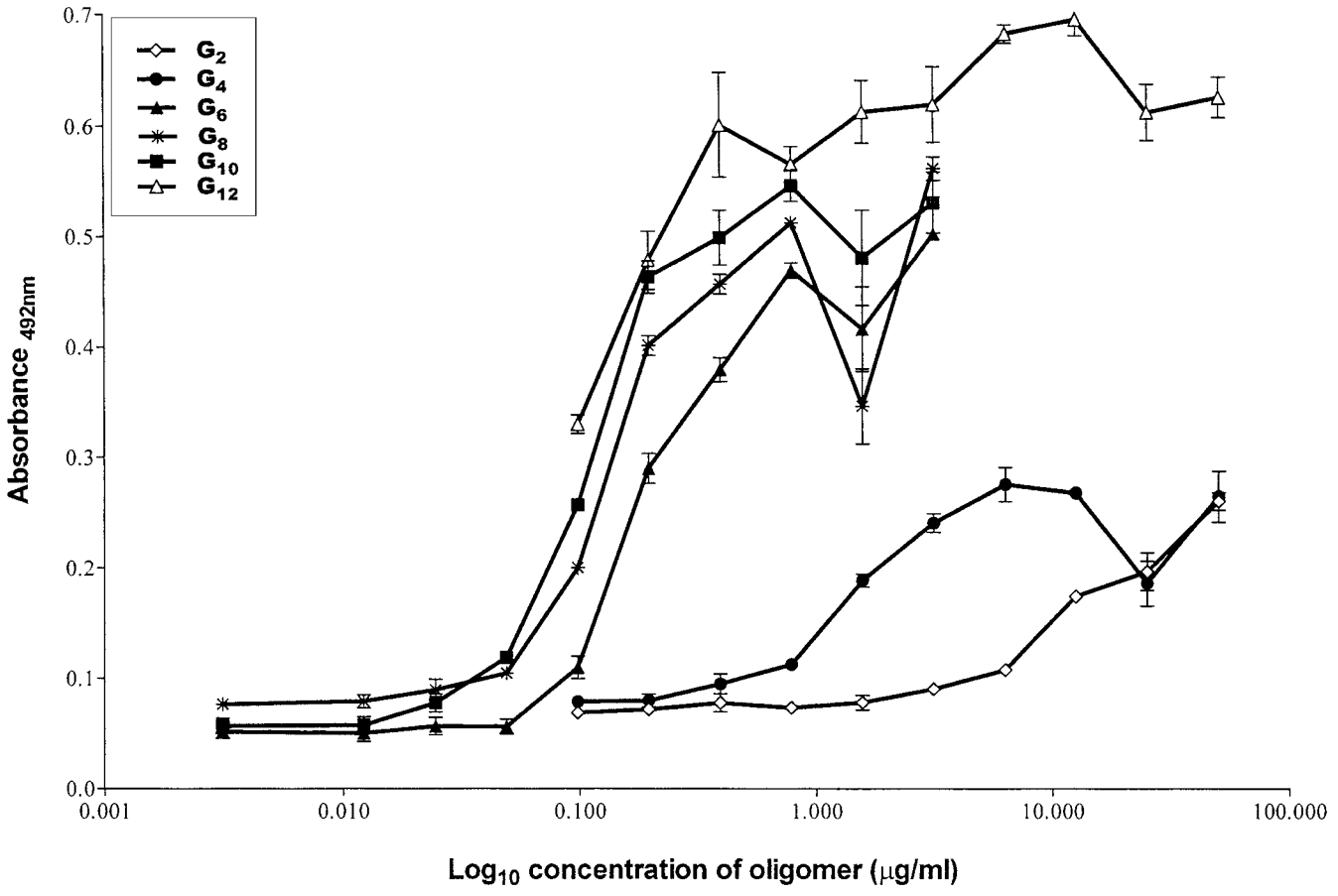

FIG. 3. Inhibition of purified antibody F9/3-3 binding to single-stranded native DNA in a competitive ELISA, by oligomers containing increasing amounts of guanine residues. Values represent the mean (SEM) of three determinations per concentration.

are removed from mammalian cells solely by NER, measurement of these lesions was used as a positive control, using a previously validated assay (30). We have previously noted the stimulation of DNA repair by hydrogen peroxide treatment of cells and used this sublethal insult to promote the removal of 8-oxodG and T<>T. Viable cell numbers per unit volume of supernatant remained constant throughout the experiment and did not differ between control and XPA cells. In contrast to the NER proficient cells, no significant increase in supernatant 8-oxodG with time was noted in the XPA cells (Fig. 4) and a similar result was seen for T<>T levels (data not shown). However, in both cell lines a co-appearance of lesion and oligomer was noted (Fig. 4). Indeed, comparison of these results with supernatant oligomer levels demonstrated a relationship between both 8-oxodG and T<>T and oligomer levels in the proficient ($r = 0.61$ and 0.67, respectively) and XPA ($r = 0.65$ and 0.97, respectively) cell lines, the latter of which was highly significant ($P = 0.007$).

Application of ELISA to Human Urine Samples

The potential application of this antibody to the detection of single-stranded oligomers in urine samples was supported by the failure of putative urinary constituents to interfere with the assay (Table 1). Validation of the competitive ELISA assay for urinary oligomers was performed. Analysis was repeated on a separate day, but with the same samples and the corrected urinary oligomers values compared ($r^2 = 0.48$, $P = 0.0003$; Fig. 5). Proof of the potential use in a repair assay derived from application of F9/3-3 in a competitive ELISA with urine from healthy, human volunteers showed appreciable inhibition (3.89–12.51%), indicating the presence of oligomers. Furthermore, oligomer levels correlated with two other urinary products of DNA damage, 8-oxodG and T<>T ($r = 0.54$ and 0.61, respectively).

DISCUSSION

The successful repair of DNA damage is an important factor in the prevention of carcinogenesis and increasing evidence suggests that polymorphisms in DNA repair enzymes may be associated with cancer risk. It is therefore essential to assess *in vivo* repair capacity, preferably noninvasively. Immunoassays

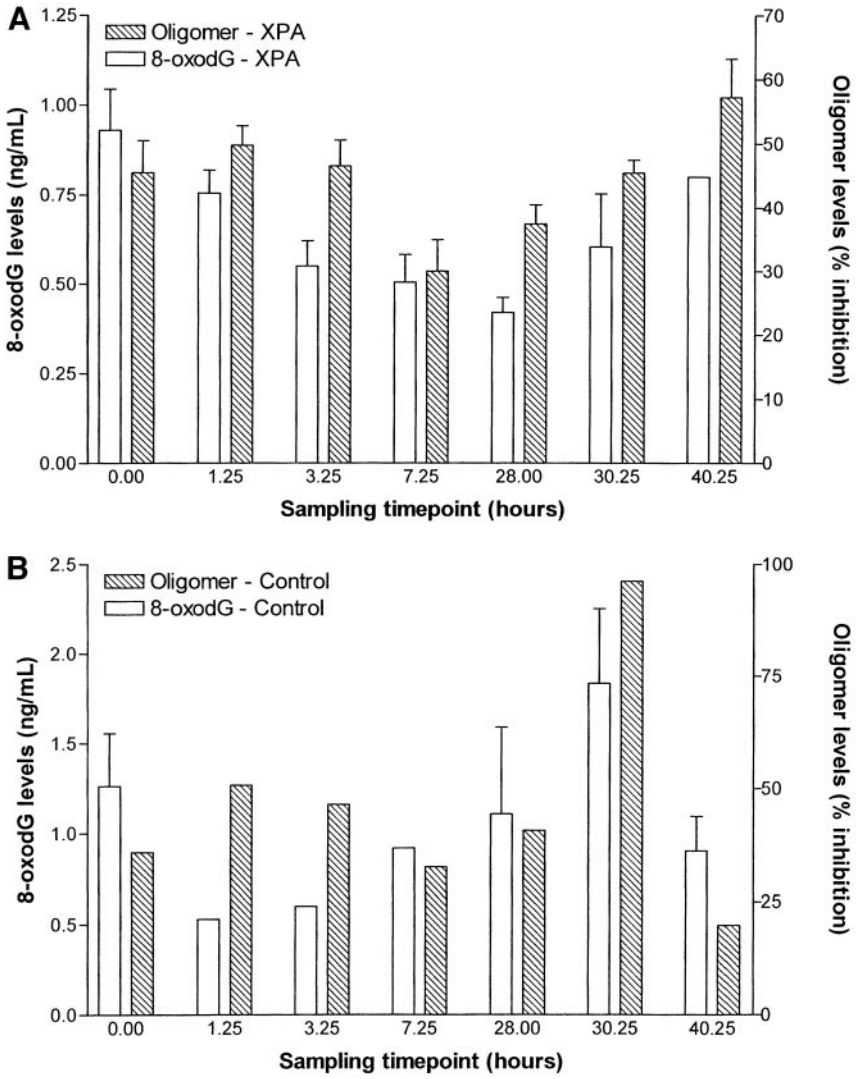

FIG. 4. Changes in 8-oxodG and oligomer levels, as determined by competitive ELISA, in NER-proficient (control) and -deficient (XPA) fibroblasts with time, following 300 μM hydrogen peroxide treatment. Bars indicate mean ($\pm$ SD of $\geq$three determinations).

represent a most appropriate method by which this may be achieved.

In order to produce monoclonal antibodies to oxidative DNA lesions, we have adopted an established procedure using a protein-bound, C-8 hydroxylated ribonucleoside as the immunogen (8-oxoguanosine (32)). However, instead of recognising the immunogen, the resultant monoclonal shows specificity for the native deoxynucleoside, requiring a minimum of two guanine moieties, although longer sequences are also recognised. This property of an antibody raised to a synthetic immunogen is shared with a monoclonal antibody derived from an autoimmune MRL/lpr mouse (33) and supports the hypothesis that oxidatively modified DNA, perhaps guanines specifically, may be an immunogen driving the autoimmune process seen in systemic lupus erythematosus (28).

Failure to recognise the immunogen is perhaps not surprising, as hydroxylation at the C-8 position represents only a minor change to the compound's chemical structure (described by Cooke *et al.* (25)), nevertheless gross alterations to nucleosides are not certain to generate lesion specific antibodies. Following immunisation with benzo[a]pyrene-diolepoxide-treated calf thymus DNA, Van der Schans *et al.* (34) noted the antibody produced by one clone specifically recognised single-stranded DNA. Monoclonal antibodies to adenosine and guanosine have previously been produced intentionally, using the ribonucleoside linked to a carrier protein (29). Although whilst suitable for the detection of single-stranded DNA, these antibodies, despite being immunised with the ribonucleoside, failed to detect RNA (29), supporting the observation by Mueller and Rajewsky (35) that periodate-linked ribo-

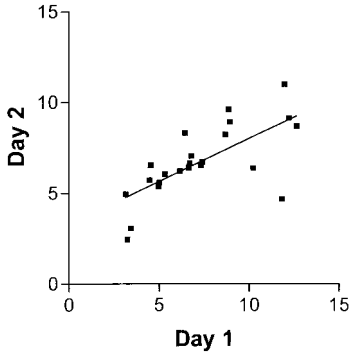

FIG. 5. Reproducibility of F9/3-3-based, competitive ELISA assay for urinary oligomers.

nucleosides more closely resemble the deoxynucleoside, justifying this approach for the production of antibodies to DNA lesions. In contrast to the monoclonal antibodies of Traincard *et al.* (29) which could detect an oligomer as short as a 40-mer at 2 μg/mL, F9/3-30 successfully detected an oligomer containing only two guanine bases, despite requiring at least 6 μg/mL. The authors proposed the use of these antibodies to the detection of contaminant single-stranded DNA in biological fluids, although such an application has not appeared in the literature. Van der Schans *et al.* (34) detected single-strand breaks in DNA by utilising alkaline unwinding of DNA in conjunction with a monoclonal antibody to single-stranded DNA, described above. This assay was then used to study global DNA repair in white blood cells whilst still in whole blood, although could not discriminate between NER, glycosylase and endonuclease actions (36).

Examination of 8-oxodG, T<>T and oligomer levels in the cell culture supernatants from NER proficient and deficient cell lines treated with hydrogen peroxide would indicate that oligomer levels are reflective of repair. This is demonstrated by the significant correlation between oligomer and T<>T levels in the XPA cells and the co-appearance of lesions and oligomers in the supernatants. Clearly not all oligomers contain 8-oxodG or T<>T and hence the poorer relationship between 8-oxodG, T<>T and oligomers in repair proficient cells. This trend between oligomers and lesions was also seen in the urine samples. However, the relationship between T<>T and oligomers was closer than that between 8-oxodG and oligomers, presumably due to the fact that all T<>T are repaired by NER, but urinary 8-oxodG can arise from other processes.

Measurement of DNA lesions in urine has generally been accepted as being entirely reflective of repair. Increasingly, however, there are reports of urinary lesions being derived from diet (37), cell death (38) and extra-DNA sources, e.g., nucleotide pool (39), confounding their accurate assessment and limiting meaningful interpretation. Measuring lesions in a manner such as to specify their source would be highly advantageous and overcome the above problems. We have previously speculated that the discrepancy in levels of 8-oxodG measured by HPLC with electrochemical detection (HPLC-EC) and ELISA methods may, in part, be due to the presence of lesions in the form of oligomers and that a monoclonal antibody to 8-oxodG has the potential to detect lesions in this form, unlike HPLC-EC (23). Herein, not only do we demonstrate the presence of urinary oligomers, but also indicate that these oligomers do indeed contain lesions, supporting a recent report for the role of NER in 8-oxodG removal (40).

We present preliminary evidence to suggest that this antibody to single-stranded DNA may be useful in the analysis of DNA repair. Positive correlations between adduct and oligomer levels in our *in vitro* model, which are also observed with *in vivo* urinary measurements support our previous hypothesis that the adducts are present, at least in part, in the short oligonucleotides (23) and are therefore highly specific targets for the investigation of DNA repair.

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