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TECHNICAL PROGRESS REPORT

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AN IMMUNOCHEMICAL APPROACH TO THE STUDY OF DNA DAMAGE AND REPAIR

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1. OBJECTIVE

The overall objective of this project is to produce antibodies to unique modified DNA bases and develop immunochemical assays to quantitate these lesions in damaged DNA. During this past year we have characterized antibodies to 8-oxopurines, produced novel antibodies to 5-hydroxyuracil and developed new methodologies to increase our level of sensitivity of detection.

2. PROGRESS REPORT

A. Properties of Antibodies to 8-Oxopurines

The deoxyribose derivatives of 8-hydroxyadenine and 8-hydroxyguanine have been found as products of the radiolysis of nucleosides and DNA. We have prepared the nucleoside derivatives of these compounds by chemical synthesis. To produce the immunogens, 8-hydroxyadenosine and 8-hydroxyguanosine were conjugated to BSA and RSA by the method of Erlanger and Beiser. Rabbits were immunized with these conjugates and polyclonal antibodies were produced.

The antibodies to 8-oxoA and 8-oxoG precipitated the homologous antigens in an Ouchterlony gel diffusion test and no cross-reactivity was observed with either antibody towards adenosine, thymidine, guanosine or thymidine monophosphate conjugates. The antibodies exhibited the same specificity for the homologous conjugate in an ELISA assay.

Antibody specificity was also examined by hapten inhibition of antibody reactivity with homologous conjugates using an ELISA assay. For anti 8-oxoA the IC_{50} for 8-oxoadenosine was 8 μM. 8-Bromoadenosine, adenosine, guanosine and inosine did not inhibit even at concentrations of 1.25 mM. Similarly, the antibody to 8-oxoG was highly specific for the eliciting hapten, with the IC_{50} for 8-oxoguanosine being 0.1 μM. 8-Methoxyguanosine also inhibited the reaction but 8-oxoguanosine was about 500 times more effective. 8-Bromoguanosine, guanosine, adenosine, thymidine glycol monophosphate or 8-oxoadenosine did not inhibit at concentrations up to 100 mM. Furthermore, antibodies to 8-oxoadenosine only reacted with irradiated poly dA and not to irradiated or unirradiated homopolymers containing dC, dA or dT. We were unable to obtain consistent results with poly dG, probably because it aggregates in solution. Thus, both antibodies appear to be highly specific for the eliciting antigen exhibiting little cross-reactivity to related unirradiated and irradiated bases.

The X-ray dose response of the production of 8-oxoadenine in poly dA irradiated in phosphate buffer was also determined. Here adducts were easily detectable at 2.5 gray. In order to standardize the ELISA signal, the amount of 8-oxoadenine produced in irradiated poly dA was analyzed by HPLC. First the polynucleotide was digested to completion with P1 nuclease and the 5' phosphates removed by *Escherichia coli* alkaline phosphatase. The resulting nucleosides were analyzed by HPLC and detectable quantities of 8-oxodeoxyadenosine were

measured at doses of 22.5 gray. These data were then used to quantify the number of 8-oxoadenine residues produced per 1000 nucleotides of irradiated poly dA using an ELISA assay. Similarly the number of 8-oxoguanine adducts was quantified by HPLC and electrochemical detection.

Figure 1 shows the production of 8-oxoadenine residues in f1 and calf thymus DNA X-irradiated in phosphate buffer. A linear production of damage was observed, with single stranded DNA giving a higher signal than duplex DNA. This difference appears to be due to a greater rate of production of damage in single stranded versus double stranded DNA rather than a relative increase in reactivity with antibody since there was no difference in antibody signal when duplex pUC19 DNA was irradiated and plated directly for the ELISA (duplex) or irradiated, denatured and then plated for the ELISA (single stranded). In all cases, 8-oxoadenine was easily detected in DNA at doses of 5 gray. Figure 2 shows the production of 8-oxoguanine in X-irradiated f1 DNA. Here the adduct was detectable at 20 gray.

B. Production of Antibodies to 5-Hydroxyuracil

5-Hydroxyuracil monophosphate was synthesized and conjugated by the carbodiimide method to BSA. Primary immunization of a New Zealand White Rabbit R584 was by multiple intradermal injections of a total of 1 mg of the conjugate mixed with an equal volume of complete Freund's adjuvant. Similar booster injections were given in incomplete Freund's adjuvant three weeks later and at three week intervals. Three bleeds were taken seven days after each booster injection. With the third bleed, specific precipitation with 5-hydroxyuridine monophosphate-RSA was observed. Cross reacting antibodies to BSA and uracil were also found. To remove non-specific antibody, columns containing uridine monophosphate-BSA have been prepared. After purification, antibody specificity will be examined by standard immunochemical techniques.

C. Assay Sensitivity

In our usual ELISA-based assay we can detect slightly better than 1 lesion per 10^5 nucleotides (for example see Figure 3). We have been trying a number of approaches to increase this sensitivity including using new methods to increase the amount of DNA bound to each well as well as chemiluminescent probes to detect the antibody - DNA complex.

To increase DNA binding to the polystyrene plates, 200 ul of poly(phe.lys) solution (2 mg per 100 ml of distilled water) was added to each microtiter plate well and incubated at room temperature overnight. The liquid in the wells was then discarded and the wells were rinsed once with distilled water. Excess liquid in the wells was removed by blowing nitrogen into the wells, the plates were then further dried in an oven at 37°C for half an hour. The dried plates were kept at room temperature for at least 3 days before use. The poly(phe.lys) treated plates are stable for at least a month without much loss in there ability to bind DNA.

When a chemiluminescent probe was to be used, opaque MicroLite 1 (Dynatech) plates were pre-treated with poly(phe.lys) and 200 ul of damaged DNA (1 ug/ml in PBS buffer) was added to each well and the plates incubated at room temperature for 1 hour. The plates were washed four times with Wash buffer followed by adding 200 ul of Blocking Buffer for 1 hour at room temperature. 200 ul of primary antibody (diluted in Blocking Buffer) was added and the plates incubated for 1 hour. The plates were then washed thoroughly with Wash buffer at least four times, and 200 ul of secondary antibody (anti-rabbit alkaline phosphatase, 1:20,000 dilution in Blocking Buffer) was added and allowed to incubate for 1 hour. The plates were then washed 4 times with Blocking Buffer, and twice with Assay Buffer. Then 100 ul of Enhancer/Substrate Solution was added for 20 minutes and the chemiluminescence readings were taken (ML 1000 Luminometer, Dynatech Laboratories) at 10 minute intervals until a maximum light output was achieved. Figure 4 shows a standard curve obtained with thymine glycol-containing DNA and anti-thymine glycol antibody. With this method we can detect 1 thymine glycol per 10^6 bp.

DNA damage can also be detected by antibodies using membrane-bound DNA. Here, Westran PVDF membranes were pre-wetted by immersing in 100% methanol. Deionized water was slowly added to reduce the methanol content to approximately 5%. The membrane was then transferred to PBS solution, incubated for 15 min. and assembled on a dot blot apparatus (Bio-Rad). Then 100 ul of damaged DNA in PBS buffer (1 ug/ml) was added to each well. After 30 min, the dot blot apparatus was subjected to vacuum (through a water suction) and the solution allowed to pass through the membrane. After suction, the membrane was re-wetted briefly in 100% methanol, transferred to PBS solution, rinsed three times with PBS-tween and then incubated in blocking buffer (0.2% Tropix I-Block Reagent, 1x PBS, 0.1% Tween 20 and 0.02% sodium azide). Following thus, the membrane was incubated with primary antibody, diluted in Blocking Buffer, for 1 hour. After incubation with primary antibody, the membrane was then washed twice with Blocking Buffer. Then anti-rabbit-alkaline phosphatase (1:20,000 dilution in Blocking Buffer) was added for 15 minutes. The membrane was then washed three times with Blocking Buffer, two times with Assay Buffer (0.1 M diethanolamine, 1 mM $MgCl_2$, 0.02% sodium azide) and incubated for 5 minutes with Nitro-Block Reagent (Tropix) followed by two washes in Assay Buffer. The membrane was then immersed in Substrate Buffer (0.24 mM AMPPD in Assay Buffer) for 30 min, sandwiched between two clear pieces of plastic or Saran Wrap and placed on top of an X-ray film (Fuji XR) for 5 minutes to obtain an image of the dot blot. Using this method, we can routinely detect better than 1 thymine glycol in 10^6 bp, and occasionally, a detection limit of 1 thymine glycol per 10^8 bp has been achieved (Figure 5).

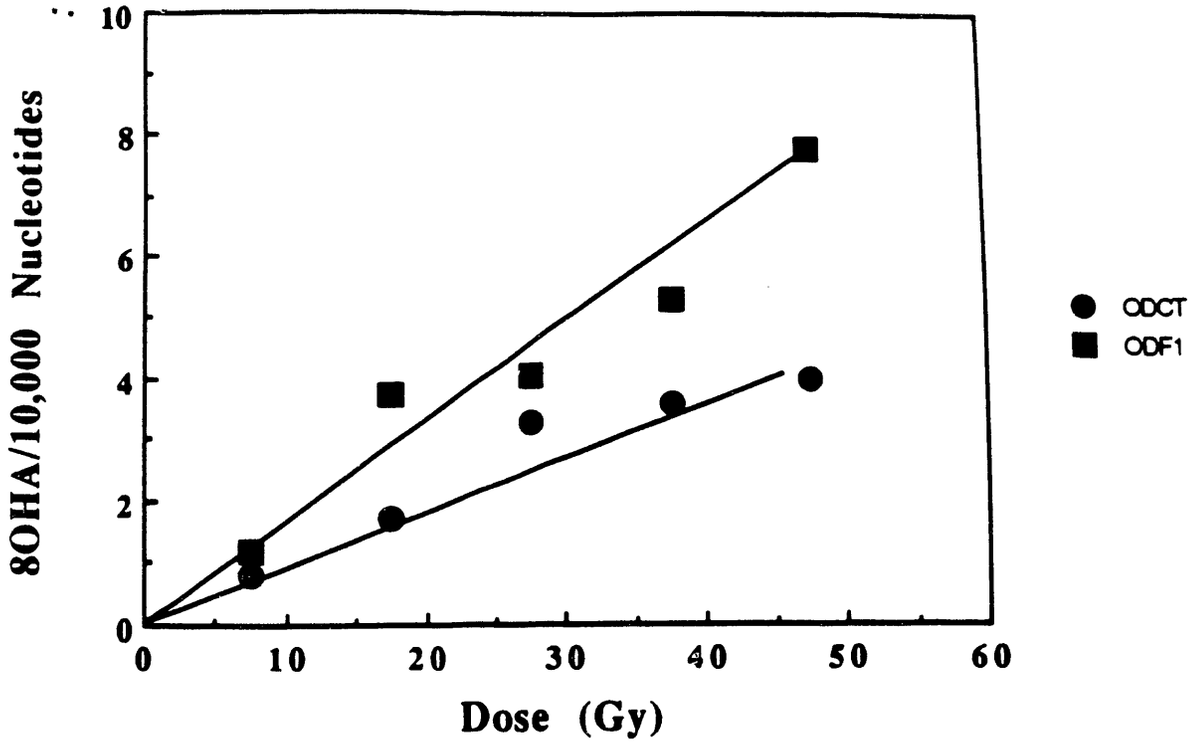

Figure 1

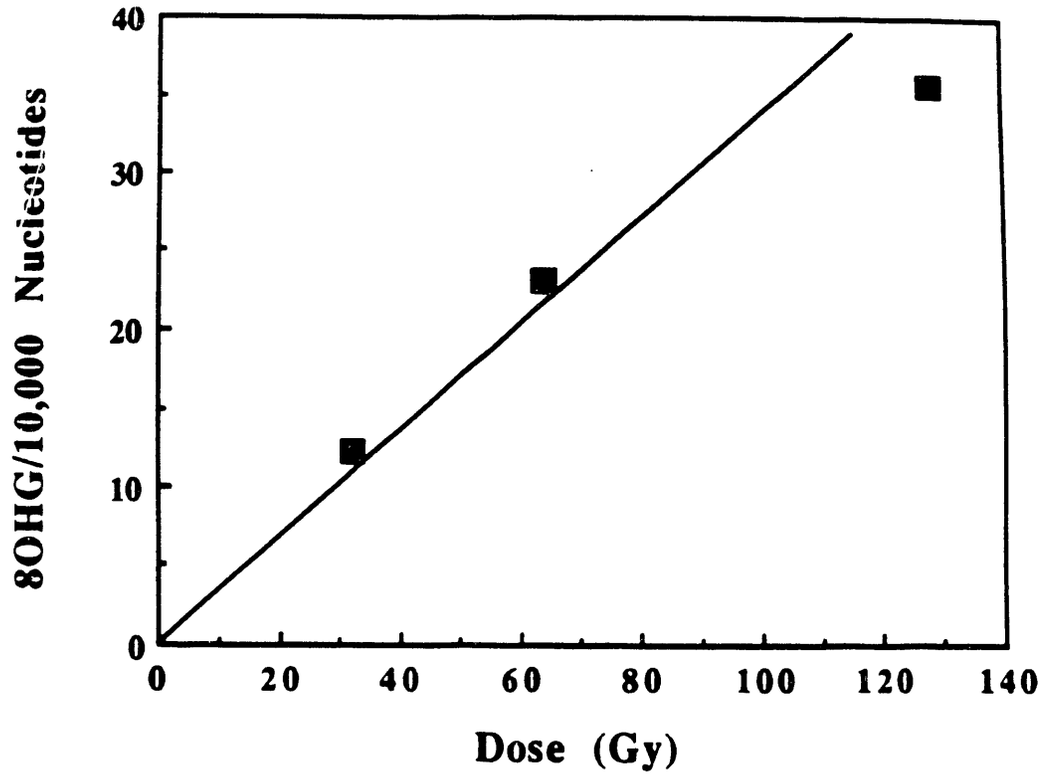

Figure 2

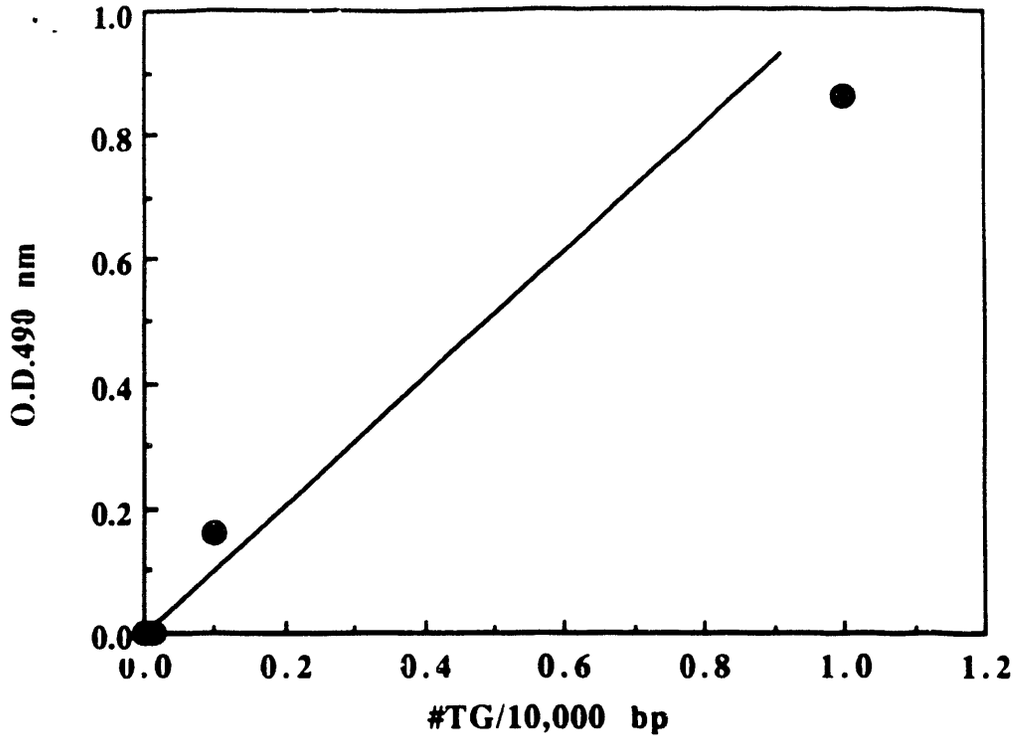

Figure 3

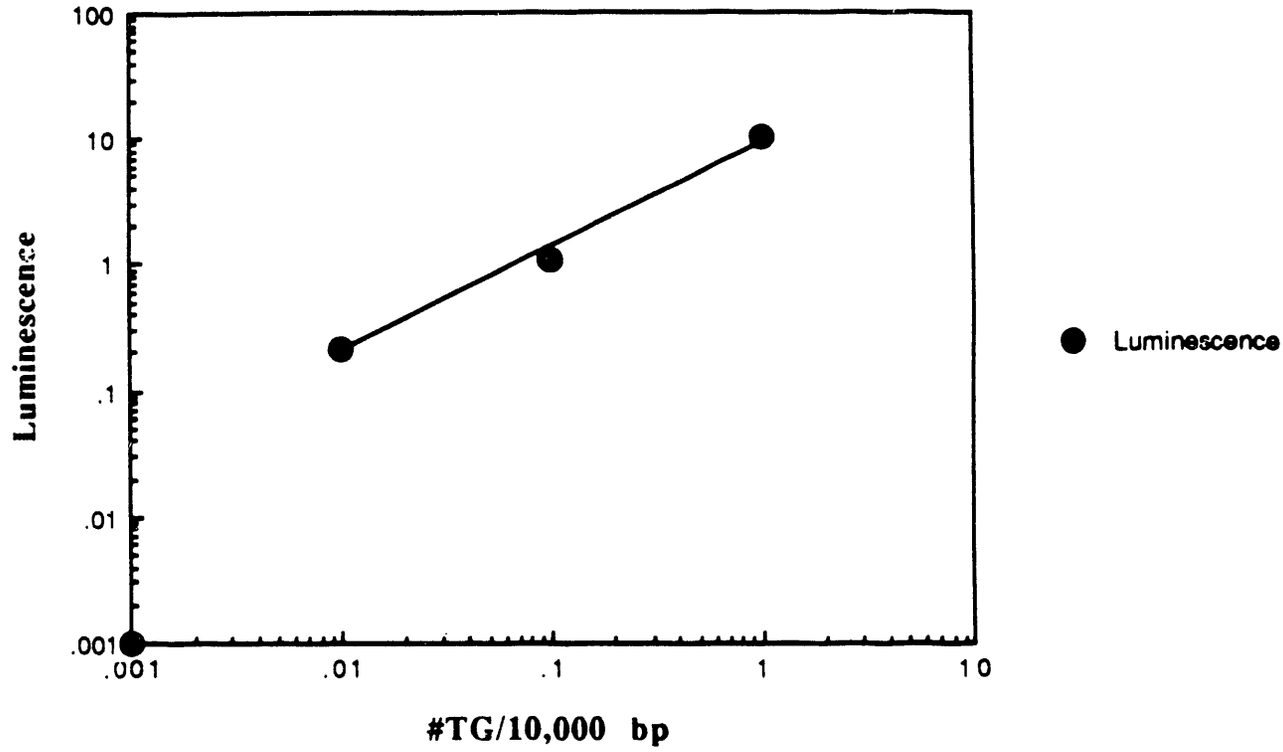

Figure 4


PBS buffer
Control (0 TG)
1 TG/10^4 bp
1 TG/10^5 bp
1 TG/10^6 bp
1 TG/10^8 bp
1 TG/10^7 bp

Figure 5

3. PUBLICATIONS

Wallace, S.S. and Ide, H. (1990). "Structure/Function Relationships Involved in the Biological Consequences of Pyrimidine Ring Saturation and Fragmentation Products", in Ionizing Radiation Damage to DNA: Molecular Aspects, S.S. Wallace and R. Painter, eds., Alan R. Liss, Inc., New York, pp. 1-15.

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ABSTRACTS

Wallace, S.S. and Ide, H. (1990). Structure/Function Relationships Involved in the Biological Consequences of Pyrimidine Ring Saturation and Fragmentation in Products. J. Cell. Biochem., Suppl. 14A, UCLA Symposia on Molecular and Cellular Biology, Abstracts 19th Annual Meetings. New York: Alan R. Liss, Inc., p. 30.

Ide, H., Chen, B.-X., Erlanger, B.F., Sterling, G. and Wallace, S.S. (1990). Immunochemical Measurement of 8-Hydroxypurines in X-Irradiated DNA. J. Cell. Biochem., Suppl. 14A, UCLA Symposia on Molecular and Cellular Biology, Abstracts 19th Annual Meetings. New York: Alan R. Liss, Inc., p.45.

4. PLANS FOR THE UPCOMING PROJECT PERIOD

Our plans for the upcoming year will be continued as originally proposed. Our primary emphasis will be to optimize our present assays to their limits of detection. We will also further characterize our antibodies to 5-hydroxyuracil and prepare new antibodies to major DNA radiolysis products such as 5-hydroxy-5-methylhydantoin and formamidopyrimidine.

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