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DNA Damage and Repair in Human Tissues

DNA DAMAGE AND REPAIR IN HUMAN TISSUES

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PREFACE

Physical and chemical agents in the environment damage the DNA of humans, and pose a major threat to human health today, and to the genetic integrity of human populations. Although studies on isolated DNA *in vitro*, on prokaryotes, on mammalian cells in culture, and on laboratory animals have provided essential background information, it is now possible to study DNA damage and repair in human tissues directly. New techniques of high sensitivity, especially those not requiring radioactive labeling have made possible quantitation of DNA damage and repair, as well as detection of residual, unrepaired DNA lesions.

In recent years, several investigators have taken up the challenge of studying damage and repair responses in humans, and we have chosen that work as the special focus of this Symposium. Major advances in understanding damage and responses in human skin, in blood cells and in human internal organs indicate three major themes. First, DNA damage levels in human tissues depend not only on the initial exposures, but also on the capapacity of that tissue for repair of the specific lesion type. Second, repair in human tissues may differ quantitatively and qualitatively from that in human cells in culture. Third, both the initial damage levels and the repair responses of humans vary over a wide range: variability is a signal characteristic of human populations!

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CONTENTS

DNA LESION MEASUREMENT IN HUMAN TISSUES

DAMAGE AND REPAIR IN SKIN

HUMAN SKIN: BIOCHEMISTRY AND MOLECULAR BIOLOGY

MODEL SYSTEMS FOR HUMAN SKIN

DNA DAMAGE AND REPAIR IN HUMAN BLOOD CELLS

DETECTION AND ANALYSIS OF HUMAN MUTATIONS

INTERNAL ORGANS

MEASUREMENT OF DNA ADDUCTS BY IMMUNOASSAYS

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INTRODUCTION

The ability to monitor for chemical carcinogen-DNA adducts in human tissues provides an indication that human exposure has occurred. Such data may eventually demonstrate the dose received and/or allow the prediction of cancer risk. At the present time evidence that the occurrence of certain adducts is associated with specific chemical exposures is accumulating, and multiple methods are being validated to confirm these observations.

Among the earliest techniques that made these studies possible are immunoassays established with antisera elicited against either individual carcinogen-DNA adducts or carcinogen-modified DNA samples (Poirier, 1984). The antisera generated are usually specific for adducts of a particular chemical class, and are not specific for unmodified DNA samples or normal nucleotides; they have been employed in radioimmunoassays (RIA) and enzyme-linked immunosorbent assays (ELISA) to measure adducts in the femtomole range, that is approximately 1 adduct in 10^7 normal nucleotides. The immunoassays are sufficiently sensitive to monitor human tissues for evidence of exposure, but are not without difficulties since their high degree of sensitivity is accompanied by high variability. In addition, cross-reactivities and other properties of the antisera may limit the capabilities of specific assays. Technical difficulties notwithstanding, there has been an impressive advancement in this field during the past several years, as more and more Laboratories are using immunoassays or combining the use of adduct-specific antisera with other techniques to determine DNA adducts in humans.

This article will focus on some technical aspects of carcinogen-DNA-adduct immunoassay development, including characteristics of antisera raised against DNA adducts and modified DNA samples, and measures which can be taken to increase immunoassay sensitivity. In addition, an example of an application of these methods for determination of DNA adducts in humans will be described; that is, studies designed to measure cisplatin-DNA adducts in peripheral white blood cell DNA and tissue DNA of patients undergoing platinum-drug based chemotherapy.

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Specificities of Antisera Elicited Against Carcinogen-DNA Adducts and Carcinogen-Modified DNA Samples

In order to elicit either polyclonal or monoclonal antisera it is necessary to couple the adduct hapten or modified DNA to a protein carrier. Adduct haptens are generally bonded covalently (10-25 molecules of hapten per molecule of protein) (Erlanger, 1980) while modified DNA samples (containing at least 1 adduct in 100 bases) are mixed with a methylated protein creating an electrostatic coupling, which is stable due to the polymeric nature of DNA (Stollar, 1980). Rabbits injected with a DNA-adduct immunogen usually respond well to repeated injections and late boosting. In contrast, a modified DNA immunogen, even if double stranded, may elicit undesirable anti-DNA antisera if the rabbits are injected many times. New antisera can be characterized by immunoassay; the most common of these are RIA (Zettner, 1973; Butler, 1980) and ELISA (Engvall, 1980), the former requiring a high specific-activity radiolabeled version of the immunogen, and the latter requiring some rather sophisticated equipment for washing and reading microtiter plates (Fig. 1, - - and ——). Competitive immunoassays can be used to test for cross-reactivity with unmodified DNA samples, normal nucleotides, the carcinogen alone and other adducts of the

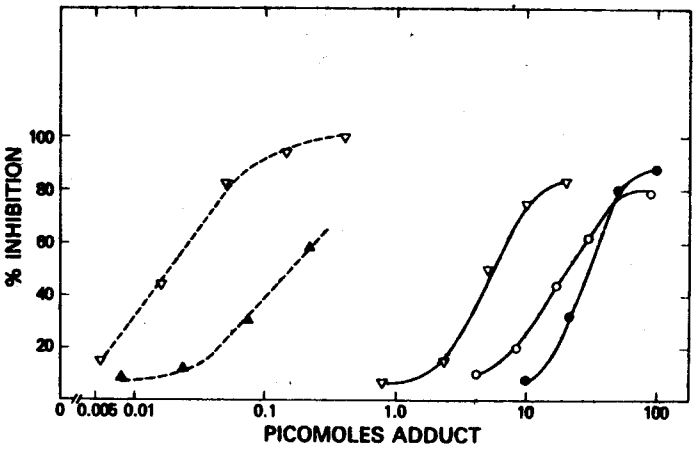

Fig. 1. Immunoassay curves established with a rabbit antiserum elicited against DNA modified with the anti-7,8 diol-9,10 epoxide of benzo(a)pyrene such that the only adduct was trans-(7R)-N²-[10 (7β,8α,9α-trihydroxy-7,8,9,10-tetrahydrobenzo[a]pyrene)-yl] - deoxyguanosine (BPdG)(Poirier et al., 1980). For RIA (——) the tracer was ^{3}H-BPdG, and for ELISA (- - -) wells were coated with denatured BPdG-DNA modified to 1% (30 pmoles/ug DNA, the original immunogen). Competition curves for RIA are: the immunogen DNA as native (0) and denatured (▽), and the individual BPdG adduct (●). Competition curves for ELISA are: the denatured immunogen (▼), and a denatured BPdG-DNA modified at 4.5 fmol/ug DNA (Santella et al., 1988) (▲).

same carcinogen as well as adducts and DNA samples modified with carcinogens of the same or different chemical classes (Fig. 1 and 2).

When an antiserum has been elicited against an individual adduct there will often be cross-reactivity for structurally-similar adducts of the same compound (Müller and Rajewsky, 1981; Groopman et al., 1984) or of other compounds in the same chemical class (Poirier, 1981; Santella, 1988). The practical implication of this is that human samples might be expected to contain a mixture of adducts from compounds of the same chemical class and therefore the numbers obtained when samples are quantified against one standard adduct may not be precise. Antisera raised against an individual adduct often recognize that adduct in a modified DNA to a lesser extent than the adduct alone (Poirier, 1981; Poirier and Connor, 1982). Therefore, it is usual to hydrolyze a biological DNA sample in order to measure all of the adducts present. In addition, an anti-adduct antiserum frequently has specificity for ribo-adducts of the same compound. Thus, removal of RNA during the preparation of biological DNA samples ensures exclusive measurement of DNA adducts (Poirier, 1981).

An antiserum elicited against a chemically-modified DNA often has little (Poirier et al., 1980) or no (Reed et al., 1990) cross reactivity for the individual DNA adduct (Fig. 1, ●—●). In this case, biological samples are generally assayed as native or denatured. Such an antiserum may recognize denatured modified DNA better than native modified DNA (Fig. 1, ○ and ▽) even though the immunogen was native (Poirier et al., 1980). Recognition of DNA samples modified with compounds of the same chemical class is frequent. For example, an antiserum raised against DNA modified with benzo(a)pyrene-7,8diol-9,10epoxide is also specific for DNAs modified with a number of polycyclic aromatic hydrocarbon-diol epoxides (Fig. 2; Weston, et al., 1989), and an antiserum raised against cisplatin-DNA recognized DNA samples modified with a number of cisplatin analogs (Lippard et al., 1983). Thus, in practice, results obtained with human samples, in which quantitation is against a specific modified-DNA standard, are not necessarily precise because of the possibility that each DNA sample is composed of a mixture of DNA adducts recognized by that antiserum. In addition, the extent of DNA modification may influence the antibody recognition (Van Schooten et al., 1987; Santella et al., 1988). To be sufficiently immunogenic a modified DNA must have adducts in the range of one adduct in 100 bases; this type of DNA sample is conformationally very different from a biological sample modified in the range of one adduct in 10^6 bases. An antiserum raised against a highly-modified DNA immunogen may not recognize all of the adducts in a DNA modified at a significantly lower level and the resulting inaccuracy will yield an underestimation of the biological samples when they are quantified against a highly-modified DNA standard curve (Van Schooten et al., 1987; Santella et al., 1988; Poirier et al., 1988). If the discrepancy between antibody recognition of high and low modified DNA samples is only several fold it may be possible to obtain accurate values by using a low-modified standard curve (see Fig. 1, ELISA curves). However, in validating the assay it is advisable to check the absolute adduct quantitation by another method, such as a radiolabeled carcinogen (Santella et al., 1988).

Factors Influencing the Sensitivities of RIAs and ELISAs for DNA Adduct Measurements in Humans

In general, the sensitivity of an RIA rests upon the affinity of the antiserum (ideally above 10^8 liters/mole) and the specific activity of the tracer (>6 Ci/mmole). For measurement of human DNA adducts the amount of DNA that can be put into one RIA tube is an important factor which must be determined for each assay. In the case of an anti-DNA adduct antiserum, hydrolysis of the DNA sample is required for quantitative determination,

and a significant increase in sensitivity can be obtained by chromatographing the hydrolysate and assaying the appropriate column fractions for adduct without the interfering normal nucleosides (Müller and Rajewsky et al., 1981; Plooy et al., 1985). The sensitivity of an RIA can be augmented somewhat through the use of sequential saturation (non-equilibrium) conditions (Zettner and Duly, 1974), in which the tracer is given a short time to compete after the antiserum and non-radiolabeled inhibitor have reached equilibrium. Often an RIA will not be as sensitive as an ELISA (see Fig. 1), but that may depend on the antiserum in question (Poirier, 1990).

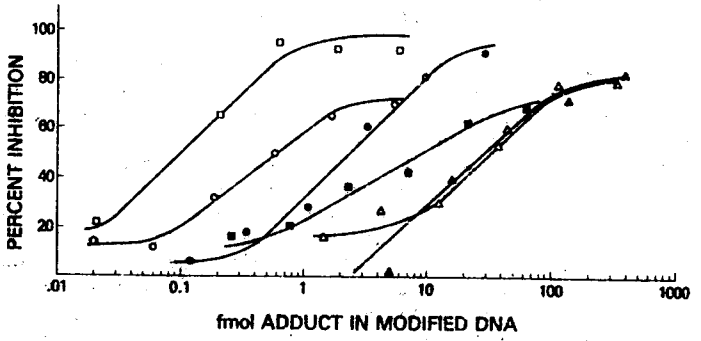

Fig. 2. Competitive ELISA with the same conditions as Fig. 1 (- - -) in which the standard immunogen BPdG-DNA is competed as denatured (0). Other curves are denatured DNA samples modified in the range of 0.1-1.0% with the diol-epoxides of : chrysene (□), benzo[k]fluoranthene (△), dibenz[a,c]anthracene (▲) and the bay region (0) and non-bay region (■) benz[a,c]anthracenes. This ELISA demonstrated that the antiserum has specificity for all of the above modified DNA samples.

For ELISAs the amount of DNA which will not alter the standard curve provides the limit of sensitivity, and can be in the range of 35-50 ug of native or denatured DNA per well (Poirier, 1990). Hydrolytic enzymes generally inhibit ELISA reactions and must be removed before assay; if this is done chromatographically and the appropriate fractions assayed an impressive increase in sensitivity can be achieved (Plooy et al., 1985). The sensitivity of an ELISA can also be augmented by the choice of enzyme substrate. For example, a 10 fold increase in the sensitivity of the benzo(a)pyrene-DNA standard curve was obtained by using methyl-umbelliferyl-phosphate as compared to p-nitro-phenylphosphate. The former compound gives a fluorescent product with a much more intense signal than the colorimetric end-point of the latter (Santella et al., 1988).

APPLICATION OF IMMUNOASSAYS FOR THE DETERMINATION OF DNA ADDUCTS IN TISSUE AND BLOOD CELL DNA OF CANCER PATIENTS

The earliest attempts to measure DNA adducts in human tissues comprised studies in which lung tumor and surrounding lung tissue were examined for evidence of polycyclic aromatic hydrocarbon-DNA adduct formation (Perera et al., 1982) using an ELISA (Fig. 1, - - -) established with the anti-BPdG-DNA antiserum (Poirier et al., 1980). Even though a small percentage of the samples were positive in the ELISA, the investigation encountered serious problems. It was not possible to document the dose of hydrocarbon received from smoking and other sources even though extensive questionnaires were administered. In addition, there was no correlation between the heaviest smokers and the individuals with the highest adduct levels, and there was a great deal of uncertainty concerning the choice of a proper control. In an attempt to overcome these difficulties and validate the use of ELISAs to determine DNA adducts in human samples, antiserum was elicited against the DNA-damaging chemotherapeutic agent cisplatin (Fig. 3). The immunogen in these studies was calf thymus DNA modified to approximately 4 adducts per 100 nucleotides with cisplatin (Poirier et al., 1982). Our intention was to determine DNA adducts in a human cohort in which the exact exposure dosage was known, and this approach had the additional advantage that it would be possible to obtain truly unexposed controls.

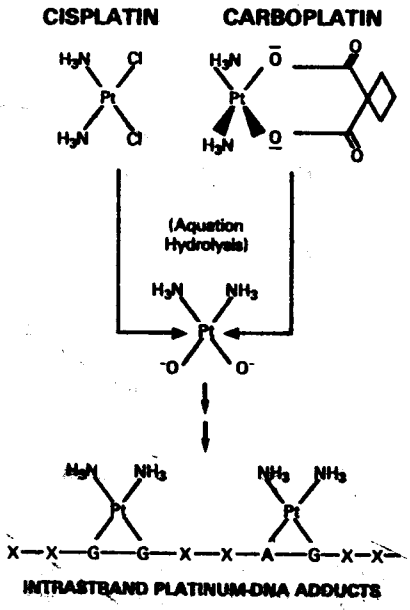

Fig. 3. Structures of cisplatin and carboplatin (CBDCA), and their major intrastrand adducts with DNA.

Cisplatin is a highly effective chemotherapeutic agent (Calvert, 1986; Einhorn et al., 1985) currently in use for a variety of human cancers, and responsible for extensive cures (approximately 90%) in individuals with testicular cancer. This compound, and other cis-reacting analogs such as carboplatin (CBDCA, Fig. 3), bind to DNA at the N7 positions of adjacent deoxyguanosines or a deoxyadenosine and a deoxyguanosine (5'-3') in the same DNA strand. Although interstrand DNA adducts are also formed, the intrastrand bidentate adducts comprise the major fraction of platinum bound to DNA (Plooy et al., 1985) and were the major epitopes recognized by the antiserum elicited against cisplatin-modified DNA (Poirier et al., 1982). In early studies this antiserum was shown to be specific for the highly-modified immunogen DNA, DNA from cultured cells exposed to cisplatin and nucleated blood cell DNA from a patient receiving cisplatin chemotherapy (Fig. 4). In addition, the antiserum was not specific for unmodified calf thymus DNA or DNA from a patient receiving non-platinum-based chemotherapy (Fig. 4). These initial studies suggested that monitoring of human tissues for DNA adducts of platinum drugs would be possible.

Cisplatin-DNA Adducts in Human Tissues Obtained at Autopsy

A variety of tissues were obtained from eight individuals autopsied at the NIH clinical center; the cohort comprised one female with breast cancer, one male with diffuse histiocytic lymphoma and six females with ovarian cancer. These patients ranged in age from 35 to 75 years and had received cumulative platinum drug doses between 700 and 9210 mg/m² of body surface area. Four of the patients with the highest cumulative drug doses

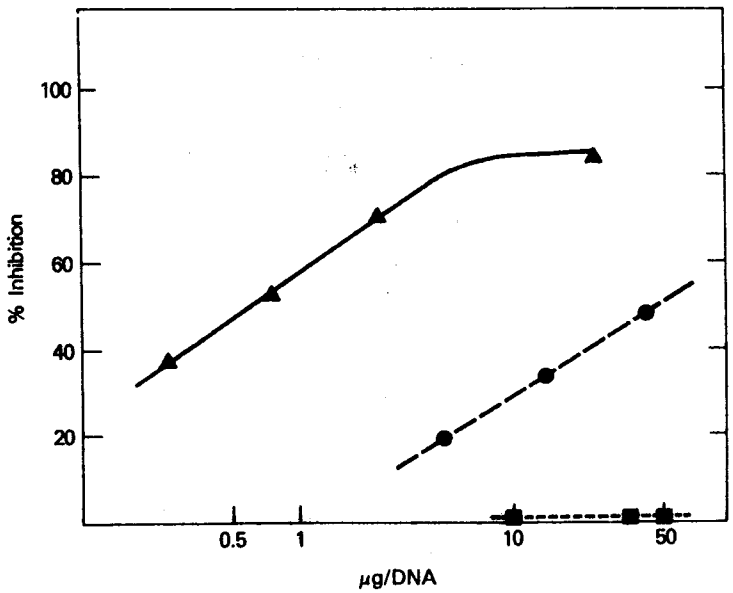

Fig. 4. Competitive ELISA with anti-cisplatin-DNA antiserum. Immunogen cisplatin-DNA was coated on wells and competed against: (▲) DNA from mouse keratinocytes exposed to 20 u molar cisplatin; (●) DNA from blood of a patient who received 40 mg/m² of body surface area of cisplatin; (■) unmodified calf thymus DNA and a DNA sample from a patient who did not receive platinum drugs.

had achieved remissions of 4-7 months duration prior to events which lead to their final demise, and 6 of the patients had received their most recent therapy between 2 and 15 months before they were evaluated for adducts. Ovarian tumor tissue was obtained on 4 of the 8 individuals, and other tissues included bone marrow, lymph node, spleen, kidney, liver, peripheral nerve and brain. Cisplatin-DNA adducts, determined by ELISA (Table 1), were found in all of these tissues, although not all tissues were present for each individual, and not all tissues of one individual contained adducts.

The results show that cisplatin-DNA adducts accumulate in a wide variety of human tissues, including tissues that are target sites for cisplatin toxicity such as kidney, peripheral nerve, brain and tumor. Adduct levels in tumor were found to be similar to those in other tissues of the same individual. The highly-persistent nature of the intrastrand bidentate adduct was demonstrated in these experiments since at least three of the individuals were autopsied 12-15 months after receiving their last therapy. In a previous study (Poirier et al., 1987) adducts were determined in kidney, liver and spleen from four individuals; one of these individuals had survived 22 months since the last therapy, and adducts were still measurable in the kidney DNA. It is possible that the chemotherapeutic efficacy, as well as the toxic effects of the platinum drugs are related to the widespread adduct distribution and the high degree of persistence observed here. These compounds are rodent carcinogens (Leopold et al.,1979; Barnhart and Bowden, 1985), and may eventually cause second tumors in cancer patients cured of their original malignancies.

Table 1. ELISA Determination of Platinum-DNA Adducts (attomoles of adduct/ ug of DNA) in Multiple Human Tissues Obtained at Autopsy

	Patient Number							
TISSUE	1	2	3	4	5	6	7	8
Tumor	-*	-	106	58	-	176	73	-
Bone Marrow	-	0	100	77	-	-	45	36
Lymph Node	0	143	-	0	-	-	-	-
Spleen	88	343	74	-	141	-	283	113
Kidney	-	511	66	50	-	315	122	184
Liver	-	457	10	45	211	342	96	78
Peripheral Nerve	-	0	-	0	-	62	0	315
Brain - White	-	143	122	62	-	-	-	833
Brain - Grey	-	306	100	112	-	-	-	456

The ELISA lower limit of sensitivity was 25 attomol/ug DNA and is designated by "0". *Specimen not available.

Cisplatin-DNA Adducts in Human Nucleated Blood Cell DNA

Since it is rarely possible to obtain biopsy materials of malignancies or normal tissues from living patients, we investigated the possibility that DNA adducts measured in nucleated blood cell DNA might vary with dose, be indicative of DNA adducts in the tumor, and reflect or predict disease response. For these studies DNA was prepared from 35-45 ml of blood taken from ovarian or testicular cancer patients at the NIH clinical center. Patients were given 5 days of cisplatin infusion and samples were drawn on the morning of day 6. Frequently samples were obtained from the same individual on multiple monthly cycles of therapy since the drug was usually given during one week, and this was followed by three drug-free weeks to comprise one cycle of treatment (Reed et al., 1986). ELISA results are shown in Fig. 5. Adduct levels in 27 positive samples were plotted as a function of cycle of treatment and the dose-related increase in adducts had a linear regression correlation coefficient of 0.79. Six samples obtained from untreated individuals were clearly negative for adduct formation (Fig. 5). What is not shown in Fig. 5 is that in any large group of samples approximately 40% of samples from treated individuals do not contain measurable adducts. However, those which are positive exhibit a dose response suggesting that adducts accumulate during monthly exposures. Since it is known that most nucleated blood cells have a relatively short life-span, it is assumed that this accumulation over weeks of time reflects

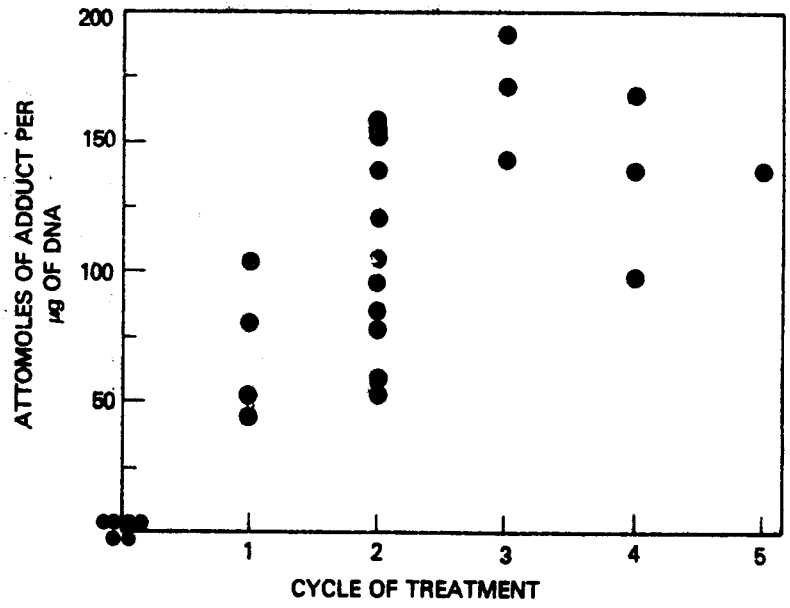

Fig. 5. Accumulation of cisplatin-DNA adducts in nucleated blood cell DNA of previously-untreated ovarian and testicular cancer patients during monthly cycles of cisplatin-based therapy. Six individuals had samples taken before treatment, serving as unexposed controls.

adduct formation which begins in the granulocyte precursor cells and continues to damage these cells with continuing cisplatin exposure.

The relationship between blood cell DNA adduct formation and disease response was addressed in 55 ovarian and 17 testicular cancer patients treated at the NIH Clinical Center (Reed et al., 1987). The ovarian patients comprised four treatment groups, two on combination chemotherapy and two receiving high-dose, single agent therapy of either cisplatin or carboplatin (Fig. 3). The testicular cancer patients all had metastatic "poor prognosis" disseminated disease and were given one of two combination protocols. Disease response was obtained from the medical records and was categorized as: complete response (CR), or total absence of visible disease; partial response (PR), or greater than 50% tumor reduction; and, no response (NR), or less than 50% tumor reduction. When blood samples were assayed by ELISA and plotted as a function of disease response (Fig. 6) there were many samples from non-responders which did not contain measurable adducts. Conversely, the median adduct level for the CRs was the highest of all three groups (Fig. 6), and decreased significantly with the poorer responses in a statistically significant trend (Reed et al., 1987). Thus, it appears that when a large group of samples is analyzed high adduct levels in blood cell DNA can correlate with a favorable disease response. This is presumably related to the fact that adduct levels in blood cell DNA also reflect adduct levels in the tumor.

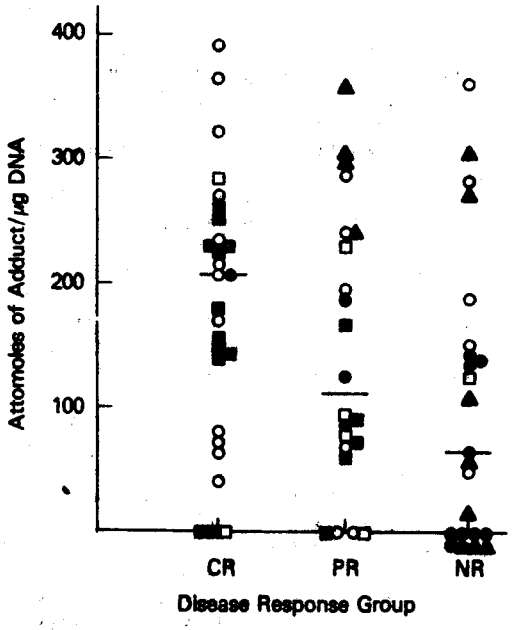

Fig. 6. Cisplatin-DNA adduct levels in blood cell DNA from 72 testicular and ovarian cancer patients plotted as a function of response to therapy. See text for definitions of complete response (CR), partial response (PR) and no response (NR). Median adduct levels for the group are designated by the solid bars.

CONCLUSIONS

These studies demonstrate the successful utilization of DNA adduct-specific antisera for determination of chemicals bound to DNA in human tissue by immunoassay. Currently, immunoassays provide incontrovertible evidence of human exposure. Data generated by these assays may help elucidate the mechanisms by which DNA binding drugs are clinically active, and may provide information useful in the molecular epidemiology of cancer.

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