exchange in cigarette smokers and non-smokers, *J. Natl. Cancer Inst.* **79,** 449–456 (1987).

D. H. Phillips, Chemical Carcinogenesis. In: *The Molecular Basis of Cancer*, eds. P. B. Farmer and J. M. Walker, Croom Helm, Beckenham, U. K., pp. 133–179 (1985).

R. M. Santella, C. D. Lin and N. Dharmaraja, Monoclonal antibodies to a benzo[a]pyrene diolepoxide modified protein, *Carcinog.* **7,** 441–444 (1986).

L. Shugart, Quantifying adductive modification of hemoglobin from mice exposed to benzo[a]pyrene, *Anal. Biochem.* **152,** 365–369 (1986a).

L. Shugart, Quantitating exposure to chemical carcinogens: *in vivo* alkylation of hemoglobin by benzo[a]pyrene, *Toxicol.* **34,** 211–220 (1986b).

P. L. Skipper, M. S. Bryant, and S. R. Tannenbaum, Determination of human exposure to carcinogenic aromatic amines from hemoglobin adducts in selected population groups. In: *Carcinogenic and Mutagenic Responses to Aromatic Amines and Nitroarenes*, eds. C. M. King, L. J. Romano, and D. Schuetzle, Elsevier, New York, pp. 65–71 (1988).

W. G. Stillwell, M. S. Bryant, and J. S. Wishnok, GC/MS analysis of biologically important aromatic amines. Application to human dosimetry, *Biomed. Environ. Mass Spectrom.* **14,** 221–227 (1987).

M. Törnqvist, J. Mowrer, S. Jensen, and L. Ehrenberg, Monitoring of environmental cancer initiators through hemoglobin adducts by a modified Edman degradation method, *Anal. Biochem.* **154,** 255–266 (1986a).

M. Törnqvist, S. Osterman-Golkar, A. Kautiainen, S. Jensen, P. B. Farmer, and L. Ehrenberg, Tissue doses of ethylene oxide in cigarette smokers determined from adduct levels in hemoglobin, *Carcinog.* **7,** 1519–1521 (1986b).

M. Törnqvist, S. Osterman-Golkar, A. Kautiainen, M. Näslund, C. J. Calleman, and L. Ehrenberg, Methylations in human hemoglobin, *Mutat. Res.* **204,** 521–529 (1988a).

M. Törnqvist, A. Kautiainen, R. N. Gatz, and L. Ehrenberg, Hemoglobin adducts in animals exposed to gasoline and diesel exhausts. I. Alkenes, *J. Appl. Toxicol.* **8,** 159–170 (1988b).

N. J. Van Sittert, G. De Jong, M. G. Clare, R. Davis, B. J. Dean, L. J. Wren, and A. S. Wright, Cytogenetic, immunological and haematological effects in workers in an ethylene oxide manufacturing plant, *Br. J. Ind. Med.* **42,** 19–46 (1985).

C. P. Wild, S. H. Lu and R. Montesano, Radioimmunoassay used to detect DNA alkylation in tissues from populations at high risk for oesophageal and stomach cancer. in: *The Relevance of N-Nitroso Compounds to Human Cancer: Exposures and Mechanisms*, IARC Scientific Publication No. 84, eds. H. Bartsch, I. K. O'Neill, and R. Schulte-Hermann, IARC, Lyon, France, pp. 534–537 (1987).

C. P. Wild, G. Smart, R. Saffhill, and J. M. Boyle, Radioimmunoassay of O^6-methyldeoxyguanosine in DNA of cells alkylated *in vitro* and *in vivo*, *Carcinog.* **4,** 1605–1609 (1983).

M. J. Wraith, W. P. Watson, C. V. Eadsforth, N. J. Van Sittert, M. Törnqvist, and A. S. Wright, An immunoassay for monitoring human exposure to ethylene oxide. In: *Methods for Detecting DNA Damaging Agents in Humans: Applications in Cancer Epidemiology and Prevention*, eds. H. Bartsch, K. Hemminki, and I. K. O'Neill, IARC Scientific Publication No. 89, IARC, Lyon, France, pp. 271–274 (1988).

Chapter

12

Development of Immunoassays for the Detection of Carcinogen-DNA Adducts

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1. INTRODUCTION

Since the mid-1970s a number of laboratories have been engaged in the quantitation and localization of DNA adducts using antisera that recognize DNA adducts and carcinogen-modified DNAs (Poirier, 1981, 1984). These antisera are sensitive probes for the presence of DNA-bound carcinogens and have proven useful in investigating mechanisms of chemical carcinogenesis in model systems (Müller and Rajewsky, 1981; Leng, 1985; Strickland and Boyle, 1984; Phillips, 1989) as well as detection of human exposure (Santella, 1988; Weston *et al.*, 1989b). The methodology that has made these studies possible evolved by adaptation of techniques previously developed for eliciting antisera against nucleosides, nucleotides, oligonucleotides, and nucleic acids. The immunogenicity of natural or unusual nucleosides covalently coupled to proteins and of nucleic acids coupled electrostatically through the negatively charged phosphates to methylated proteins had been clearly demonstrated (Stollar, 1980). The quantitative immunoassays developed with these antisera were highly sensitive and able to detect, for example, unusual bases in nucleic acids constituting a minute fraction of the total nucleotides

(Munns and Liszewski, 1980; Sawicki *et al.*, 1976). The adaptation to detection of chemical carcinogen-DNA adducts occurring at frequencies of 1 adduct in 10^5 bases or lower required primarily refinements and adaptations of the immunoassays above (Poirier, 1984; Müller and Rajewsky, 1981; Leng, 1985; Strickland and Boyle, 1984).

The focus of this chapter will be on the methodologies required to conjugate immunogens, immunize rabbits, establish immunoassays, characterize the antisera, and assay biological samples for the presence of carcinogen-DNA adducts. Included is a short discussion concerning problems that relate to standardization of immunoassays among different laboratories and a consideration of studies combining the use of adduct antisera with methods providing independent chemical identification.

2. PREPARATION OF IMMUNOGENS AND IMMUNIZATION

In order to achieve a sufficiently strong immunogenic stimulus, whether imunizing rabbits for polyclonal antisera or rodents for monoclonal antisera, it is necessary to couple the adduct hapten or the modified DNA to a protein carrier. Adduct haptens are generally bonded covalently (Erlanger, 1980), while modified DNAs are mixed with a methylated protein creating an electrostatic coupling between the nucleic acid phosphates and the protein methyl groups (Stollar, 1980; Plescia and Braun, 1967). Because of the polymeric nature of nucleic acids, this type of association is sufficiently stable to induce antibody formation. Standard covalent coupling procedures take advantage of available reactive moieties on the protein such as α-amino groups, sulfhydryl groups, phenolic hydroxyl groups, the imidazole group of histidine or the ϵ-amino group of lysine (Erlanger, 1980). The most common protein carriers are the serum albumins, hemocyanin, immunoglobulins, and ovalbumin. The serum albumins are soluble, while hemocyanins have a high molecular weight and are considered quite insoluble (Erlanger, 1980).

When coupling a nucleoside or nucleotide adduct the desired recognition site (the hapten), which is usually the chemical carcinogen bound to the base, should be distal from the site of protein attachment (Stollar, 1980). Frequent coupling sites are the 2′ and 3′ hydroxyls of ribose. Two common procedures are periodate oxidation of both 2′ and 3′ hydroxyls followed by condensation of the dialdehyde on lysine (Erlanger, 1980), and succinylation of a ribose 2′ hydroxyl group with succinic anhydride and coupling of the resulting ester to protein with carbodiimide (Stollar, 1980). Coupling can also be achieved through a 5′ phosphate by formation of phosphoramide conjugates through protein amino groups with water-soluble carbodiimides.

A deoxyribonucleoside lacking a 5′ phosphate can be oxidized on the 5′ hydroxyl to a carboxyl group and linked to a protein by water-soluble carbodiimide (Stollar, 1980).

For the production of polyclonal antisera in rabbits the ratio of hapten to protein carrier was first investigated by Landsteiner, who concluded that with serum albumin as carrier, 10 haptenic groups per protein molecule gave the optimum antigenic stimulus (Erlanger, 1980). Concentrations between 8 and 25 haptens per protein molecule have been reported by others to produce good antisera (Erlanger, 1980). Carcinogen-DNA adduct immunogens are no exception, and high-titer antiserum was first obtained against guanosin-(8-yl)-acetylaminofluorene conjugated to bovine serum albumin that contained 17–22 adduct haptens per molecule of protein (Poirier *et al.*, 1977). In general, 0.2–0.5 mg of adduct conjugated to bovine serum albumin can be injected once a week for 3–4 weeks either as bolus intramuscular injections and/or as a series of smaller intradermal injections (Stollar, 1980). Bleedings should begin one week after the last injection, and animals can be bled once a week for at least three months. If the titer starts to decline, intramuscular or intravenous boosting should restore it. Modified-DNA immunogens have produced a good antigenic stimulus with DNAs modified between 1% and 5% (1–5 adducts per 10^2 bases) mixed with an equal weight of methylated protein (Poirier, 1981). Each rabbit can be injected weekly with approximately 2 mg of the mixture (Poirier, 1984; Poirier *et al.*, 1980). The weekly injections should be stopped after 3 weeks because additional injections may elicit antisera specific for DNA (Poirier, unpublished). Similarly, boosting of animals injected with a modified-DNA immunogen may not be successful because it produces anti-DNA antibodies. A native DNA immunogen is less likely to produce anti-DNA antisera than denatured DNA. However, antiserum elicited against modified native DNA may still preferentially recognize modified denatured DNA (Fig. 3, radioimmunoassay; Poirier *et al.*, 1980).

For the production of monoclonal antibodies similar principles apply as in the case of polyclonals, but the details vary. Mice have been successfully immunized with 0.1–0.2 mg of adduct on days 1 and 21 and sacrificed on day 26 (Stollar, 1980). Usually injections are subcutaneous or intraperitoneal (Hertzog *et al.*, 1983). If only small quantities of immunogen are available, 20 μg of immunogen can be absorbed onto aluminum hydroxide and injected intradermally in Freunds adjuvant (Strickland and Boyle, 1984). If the titer is insufficient, boosters can be administered at 3–4 week intervals (Stollar, 1980; Hertzog *et al.*, 1983). Particular success in obtaining highly avid rat monoclonals has been achieved using keyhole limpet hemocyanin as protein carrier and absorbing it onto aluminum hydroxide before injection at several intradermal sites (Müller and Rajewsky, 1981). Selecting and screening of clones can be accomplished according to established procedures, as described

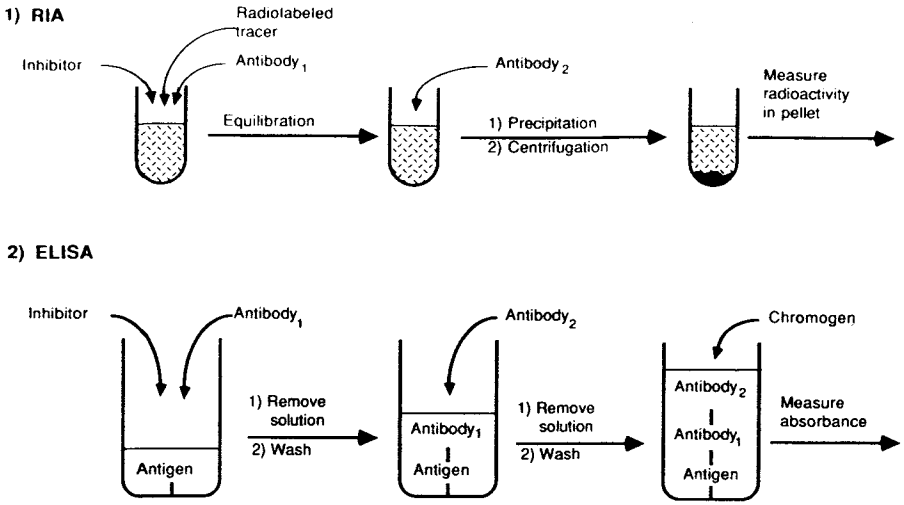

Figure 1. DNA-adduct determination by competitive immunoassay. For RIA, the radiolabeled tracer is more likely to be an adduct than a modified DNA; the inhibitor can be a standard, chemically synthesized adduct for DNA, as well as digested or undigested biological sample; antibody 1 is adduct- or modified-DNA-specific and antibody 2 can be anti-immunolglobulin or any other reagent that will precipitate the antigen-antibody complex. For ELISA, the antigen coated on the microtiter well can be either adduct or modified DNA; the inhibitor and antibody 1 are the same as for RIA; antibody 2 is an antiimmunoglobulin conjugated to an enzyme; the chromogen is a substrate for conjugated enzyme which is cleaved to a product measurable by spectrophotometry, fluorometry, radioactivity, or some other method of detection.

in Strickland and Boyle (1984). In practical terms, both polyclonals and monoclonals can be obtained with sufficient avidity to measure carcinogen and modification in biological samples. Polyclonals require less work, but monoclonals can be elicited with smaller amounts of immunogen, and once a clone is established the supply of antibody is virtually unlimited.

3. COMPETITIVE IMMUNOASSAYS

This section will describe radioimmunoassay (RIA) and enzyme-linked immunosorbent assay (ELISA) as prototypes for many immunoassay variations (Fig. 1). For clarity only one version of each type of assay will be discussed; however, assays that can be performed vary with the end point detected or the method of execution. The basic principles and problems remain the same in spite of the different variations. Both assays have in common the isolation of an antigen–antibody complex, where the antibody is bound either to an antigen on the bottom of a microtiter well (ELISA) or to a highly radioactively labeled antigen (RIA). Direct binding of antigen to antibody is useful in titering antiserum, but for the most consistent and

sensitive results it is necessary to use these assays in a competititve mode (Fig. 1). That is, increasing concentrations of unlabeled (RIA) or unbound (ELISA) competitor are used to compete for antibody binding to the radiolabeled tracer (RIA) or immunogen binding to the bottom of the microtiter well (ELISA). In both assays the competitor is either a standard immunogen, used in increasing concentrations to construct a standard curve, or an unknown sample, which is quantitated by comparison with the known standard. The following sections will describe each assay separately.

Competitive RIA (Mayer and Walker, 1980; Butler, 1980; Zettner, 1973; Zettner and Duly, 1974) involves a concentration-dependent competition for an antibody haptenic site by two chemically identical immunogens (Fig. 1), one of which is highly radiolabeled and designated the "tracer." The tracer is a reporter molecule used in a constant quantity in every tube of the assay. The RIA sensitivity will depend, in part, on the specific activity of the tracer, which should be above 10 Ci/mmol for determination of carcinogen–DNA adducts in biological samples. The isotope can be ^{3}H, ^{125}I, ^{131}I or ^{32}P, but the unstable radionuclides are generally less convenient than ^{3}H. The nonradioactive hapten or competitor can be either a standard immunogen or biological sample and is designated the "inhibitor." It is used in increasing concentrations (in different tubes) to compete for antibody (antibody 1, Fig. 1) and inhibit the antibody–tracer binding. The reaction of tracer and/or inhibitor with antibody is usually allowed to reach equilibrium, for example, at 37°C for 90 min or overnight at 4°C. The antigen–antibody complex is then separated from the whole mixture, and the radioactivity bound to antibody is measured. Separation is usually achieved with second antibody (anti-IgG, Fig. 1, antibody 2), high salt (NH_4SO_4), polyethylene glycol, protein A, or filters (Mayer and Walker, 1980; Butler, 1980). In the absence of inhibitor, a maximum amount of tracer will be found in the antigen–antibody complex, and this is termed B_0. As increasing concentrations of inhibitor are added, less and less radioactive tracer will become bound to the antibody because of inhibitor competition for the haptenic site. The degree of competition for antibody–tracer binding exhibited by each concentration of inhibitor can be expressed as a percentage (%Inhibition) of the ratio of the reduction in radioactivity at a particular inhibitor concentration and the radioactivity with no inhibitor (B_0):

$$\%\text{Inhibition} = \frac{B_0 - X_c}{B_0} \times 100$$

where X_c = radioactivity bound at inhibitor concentration c.

If the %Inhibition is plotted on a linear scale as a function of the inhibitor concentration on a logarithmic scale, the resulting curve will be linear in the

range of 20–80% Inhibition. Each assay should contain a standard curve that is constructed with increasing concentrations of immunogen and consistent (± 5–7%) for assays performed on different days. Unknown sample quantitation is achieved by comparison of the %Inhibition found from a biological sample with the inhibitor concentration at the same %Inhibition on a linear part of the standard curve. The adduct quantity is assumed to be the same in both at a given %Inhibition. This is a simple procedure for calculating RIA data but many other approaches can be used (Mayer and Walker, 1980; Zettner, 1973).

In general, RIA is a reliable and consistent assay that can be performed easily and inexpensively. There are several aspects of the assay that influence sensitivity (Müller and Rajewsky, 1981; Mayer and Walker, 1980; Butler, 1980; Zettner, 1973), and these include the antibody affinity, the specific activity of the tracer, the antibody dilution, the use of non-equilibrium incubation conditions (Zettner and Duly, 1974), the physical state of the biological sample DNA (native, denatured, or digested), and the amount of the sample that can be assayed without altering the standard curve. Sufficient sensitivity to assay for human DNA adducts generally requires antisera with affinity constants in the range of 10^8–10^9 L/mol and RIA tracers with specific activity ≥ 10 Ci/mmol (Müller and Rajewsky, 1981; Munns and Liszewski, 1980). The greater the dilution of the antiserum, the more sensitive the assay is likely to be. A common dilution range for rabbit antiserum is 1:500 to 1:2500. When titering the antiserum, B_0 will decrease linearly almost to background levels across a series of dilutions. The dilution chosen should be on the linear portion of the dilution curve and should produce tracer–antibody binding levels that are at least 4-fold above background, that is, a signal-to-noise ratio of 4:1. As described in the previous paragraph, the RIA is usually allowed to reach equilibrium for 90 min or longer before separation of the antigen–antibody complex (Zettner, 1973). However, an increase in sensitivity can be obtained using sequential saturation or non-equilibrium conditions (Zettner and Duly, 1974). Sequential saturation involves a brief tracer incubation immediately prior to separation of the antigen–antibody complex. In measuring DNA samples from biological sources, the assay sensitivity will also depend on the amount of DNA that can be introduced into the assay without alteration of the standard curve. An antiserum elicited against a DNA adduct recognizes adducts better than modified DNAs, therefore the DNA must be digested in order to measure all of the adduct present (Fig. 2; Poirier, 1981; Poirier and Connor, 1982). RIA tubes can often contain as much as 30 μg hydrolyzed DNA and accompanying hydrolytic enzymes without significantly affecting the assay. It is possible to remove the enzymes by boiling and centrifugation, although some adducts may be thermally labile.

The basic principles of competitive ELISA (Mayer and Walker, 1980; Butler, 1980; Wisdom, 1976; Engvall, 1980; Klotz, 1982; Trivers *et al.*, 1983; Harris *et al.*, 1982) are similar to those of RIA, but the constant component, the tracer, has been replaced by an immunogen bound to the bottom of microtiter plate wells (Fig. 1, antigen). The immunogen coated on the wells can be either a modified DNA or an adduct coupled to a protein (albeit a different protein than that used for immunization) (Trivers *et al.*, 1983; Harris *et al.*, 1982). Control wells are coated either with unmodified DNA alone or the carrier protein alone. Before adding serum, coated microtiter plates are blocked with protein (animal serum or casein) to cover any unoccupied sites on the plastic that might bind the antibody nonspecifically (Trivers *et al.*, 1983; Harris *et al.*, 1982). Then, in the competition step, adduct- or modified DNA-specific antiserum (diluted 1:10,000–1:200,000) is incubated with increasing amounts of inhibitor in the microtiter wells, and

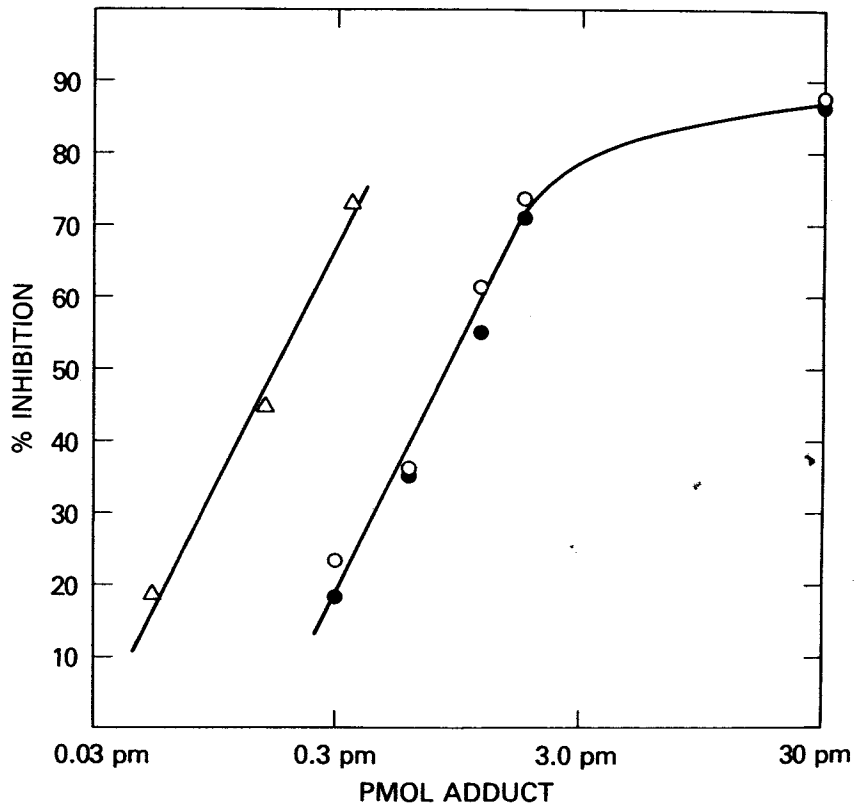

Figure 2. RIA standard curves for deoxyguanosin-(8-yl)-acetylaminofluorene in which the tracer is tritiated adduct, the antiserum was elicited against the protein-bound adduct (Poirier *et al.*, 1977), and the inhibitors are native (●), and denatured (○), and S_1 nuclease-hydrolyzed (△) claf thymus DNA modified to 1% (30 pmol/μg DNA) with *N*-acetoxy-acetylaminofluorene.

TABLE 12–1. BPDE–DNA Adduct (fmol Adduct/μg DNA), Determined by Radiolabeling and Immunoassay, in DNA from Primary Mouse Keratinocytes Exposed to ^{3}H-BP*

Radiolabeling	*p*-nitro-phenyl phosphate ELISA	*m*-umbelliferyl-phosphate ELISA	USERIA
5.6	5.12 ± 1.33	6.2 ± 2.2	5.54 ± 1.38

*Immunoassay quantitation was accomplished by comparison with a standard BPDE–DNA modified to 4.5 fmol adduct/μg DNA.
Source: Santella *et al.*, 1988a.

at equilibrium the unbound mixture is removed by washing. The next step is incubation with an enzyme-conjugated second antibody that binds to the first antibody attached inside the wells (Fig. 1, antibody 2). An enzyme commonly used is alkaline-phosphatase (as in Table 1), which can cleave phosphorylated substrates to colorimetric (e.g., *p*-nitrophenol; Harris *et al.*, 1982) or fluorometric (e.g., methyl-umbelliferone; Kriek, *et al.*, 1984) end points (Fig. 1, chromogen). The product will be present in a quantity directly proportional to the original amount of antibody bound to the microtiter well. Variations on this assay include the use of different enzyme-substrate combinations (Kabakoff, 1980), a biotin-avidin step, which amplifies the signal (Roberts *et al.*, 1986), or the use of a radiolabeled substrate, as in the ultrasensitive enzyme RIA (USERIA; Trivers *et al.*, 1983; Harris *et al.*, 1982). Like the RIA, ELISA is an inverse assay; the wells with the lowest concentration of inhibitor have the highest amount of antibody bound and the most intense end point. Data can be processed in a fashion similar to that described for the RIA.

Automatic procedures for washing and reading solutions in microtiter plate wells and the lack of necessity for a radiolabeled tracer have made ELISA somewhat more convenient than RIA. In addition, ELISA can be more sensitive than RIA (Fig. 3). However, these advantages are often offset by greater intra- and interassay variability. In addition, this highly sensitive assay is executed in several steps; therefore troubleshooting can be difficult. Like RIA, ELISA sensitivity for the measurement of DNA adducts or modified-DNA samples is limited by the quantity of DNA that can be assayed in an individual microtiter well. In many cases 50 μg of native or denatured DNA can be added to each well without altering the standard curve, but this quantity may affect the intensity of color development (Perera *et al.*, 1982). Therefore it is important to use the same amount of DNA in all wells of an assay, including the standard curve, to eliminate this source of variability. The amount of carrier DNA that can be tolerated in the assay should be determined for each new antiserum.

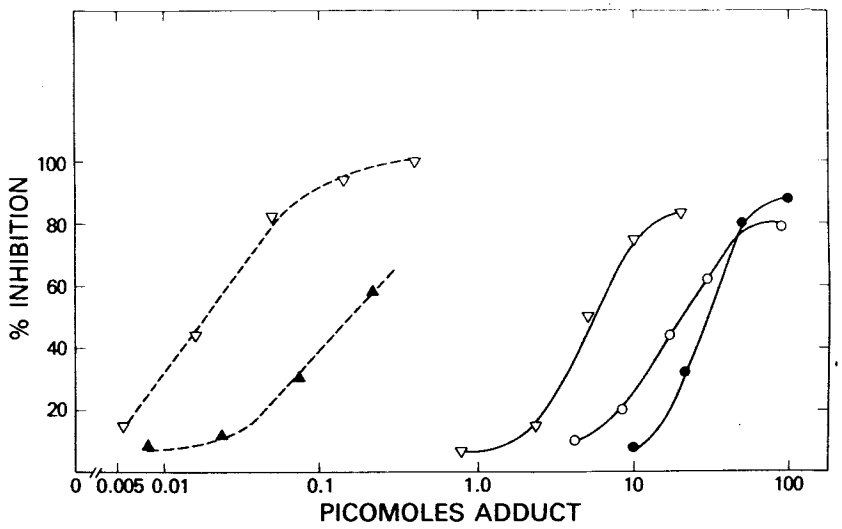

Figure 3. RIA (continuous line) (Poirier *et al.*, 1980) and ELISA (dashed line) (Santella *et al.*, 1988a) curves obtained with anti-BPDE-DNA. For RIA the tracer was tritiated BPdG[2], and for ELISA, wells were coated with denatured BPDE-DNA (the original immunogen) modified to 1% (30 pmol/μg). Competition curves for RIA are the immunogen DNA as native (○) and denatured (▽), and the individual BPdG adduct (●). Competition curves for ELISA are the denatured immunogen ▽) and a denatured, standard BPDE–DNA modified at 4.5 fmol/μg DNA (▲) (Santella *et al.*, 1988a).

4. CHARACTERIZATION OF ANTISERA

Extensive characterization of antisera is extremely important for all immunoassays. An antibody is a tool, and it is essential to know what that tool recognizes in order to interpret the experimental results. When first titering a new serum, it is customary to use a direct assay in which varying concentrations of antiserum from each bleed are incubated with excess antigen. Rabbit antisera titering should start at 1:100 for RIA and 1:1000 for ELISA. Over a range of dilutions a linear decrease in bound tracer (RIA) or end-point signal (ELISA) will be observed with decreasing antibody. A dilution in the middle of this linear range should be chosen for further competitive assays (Zettner, 1973; Wisdom, 1976). The original immunogen can then be competed over a wide range of concentrations to determine how much is required to inhibit either tracer–antibody binding (RIA) or microtiter well–antibody binding (ELISA). The most sensitive assay conditions can be achieved by decreasing the antiserum concentration and the constant immunogen (either the tracer for RIA or amount coated on microtiter plate wells for ELISA) to give the best competition with unlabeled (RIA) or unbound (ELISA) standard immunogen. Once a consistent standard curve is estab-

lished, antibody specificity for other adducts or other modified-DNA samples can be determined by competitive inhibition in the same assay.

Cross-reactivity for unmodified DNA, normal DNA bases, and the carcinogen alone should be thoroughly investigated. In the case of a chemical–DNA adduct immunogen there will often be cross-reactivity for other related chemical–DNA adducts, including different adducts of the same carcinogen (Rajewsky *et al.*, 1980; Groopman *et al.*, 1984) and carcinogen–DNA adducts formed by compounds of the same chemical class (Poirier, 1981; Santella, 1988). Antisera elicited against a specific adduct often recognize the same adduct in modified DNA to a lesser extent, and that recognition may change if the DNA is native or denatured (Poirier, 1981; Poirier and Connor, 1982). In the case of a modified-DNA immunogen, cross-reactivity with individual adducts may be (Fig. 3, ●) but is not always seen (Reed *et al.*, 1991). Recognition of DNA samples modified with compounds of the same chemical class is frequent (Fig. 4; Poirier, 1982; Weston *et al.*, 1989a; Lippard *et al.*, 1983). In addition, the extent of modification may influence the antibody recognition (Fig. 3, ELISA). To be sufficiently immunogenic, a modified DNA must have adducts in the range of 1 adduct in 10^2 bases, and this type of DNA sample can be expected to be conformationally different than a biological sample modified in the range of 1 adduct in 10^6 bases. An antiserum elicited against a highly modified immunogen may not recognize all of the adducts in a DNA modified at a significantly lower level (van Schooten *et al.*, 1987; Santella *et al.*, 1988a), and the resulting underestimation will yield inaccurate adduct values, as discussed below.

5. QUANTITATION OF DNA ADDUCTS IN BIOLOGICAL SAMPLES

Whatever the source of biological sample (cultured cells, animal tissues, or human material), isolation of DNA is a necessary prerequisite for DNA-adduct analysis. The three types of DNA preparation used most frequently for these studies are CsCl buoyant density gradients (Flamm *et al.*, 1967), chloroform and phenol extraction with RNAse incubation (Bohr *et al.*, 1985), and proteinase K digestion followed by high salt extraction and ethanol precipitation (Miller *et al.*, 1988). In theory, any DNA extraction procedure that does not chemically alter the adducts and that gives RNA-free DNA should be appropriate. Since many adduct antisera recognize RNA adducts it is important to remove the RNA for exclusive measurement of chemical-DNA adducts (Poirier, 1981). The quantities of DNA required for immunoassays depend on the degree of modification of the DNA and the sensitivity of the specific assay. For cultured cells and animal tissues 50–100 μg should be enough to assay in duplicate or triplicate on two or more occasions. This

amount of DNA can often be obtained from 10^8 cells or 100–200 mg of tissue. For human samples a minimum of 450 μg would be required to assay 50 μg of DNA per well in ELISAs on two separate occasions. This can readily be obtained from 40 ml of blood or 500 mg of human tissue.

Most antisera elicited against individual adducts do not recognize modified-DNA samples equally well (Poirier, 1981; Fig. 2). Therefore biological DNA samples generally require digestion prior to assay (Poirier and Connor, 1982). A chromatography step, inserted before the immunoassay, may remove the hydrolytic enzymes and at the same time separate the adducts for individual determination by immunoassay (Rajewsky *et al.*, 1980; Plooy *et al.*, 1985). If a biological DNA sample is likely to contain many adducts that cross-react with the same antibody, this may be the only way to obtain adduct specificity. It has the added advantage of increasing sensitivity since the amount of DNA that can be measured in one sample is virtually unlimited.

When using an antiserum elicited against a modified DNA, individual adducts are recognized less well than undigested DNA (Fig. 3, RIA), and therefore enzymatic hydrolysis of the biological samples is not necessary (Poirier, 1981; Poirier *et al.*, 1980; Santella *et al.*, 1988a). Other aspects of the immunoassay that require attention pertain to antibody recognition of native and denatured DNA (Fig. 3, RIA; Poirier, 1981; Santella, 1988; Poirier *et al.*, 1980) and the degree to which the antiserum recognizes DNA samples modified at levels lower than the original immunogen (Fig. 3, ELISA; van Schooten *et al.*, 1987; Santella *et al.*, 1988a). Competition with native and denatured immunogen DNAs should indicate whether or not the antibody recognizes one or the other preferentially. This appears to be a property of the antibody rather than the original immunogen. For example, some antisera elicited against native BPDE-DNA only recognize all of the adducts in denatured BPDE-DNA samples (Poirier *et al.*, 1980; Santella *et al.*, 1988a), while an antiserum against cisplatin-DNA recognizes native and denatured DNA samples equally (Fichtinger-Schepman *et al.*, 1985; Poirier, unpublished). BPDE-DNA is DNA modified with BPDE I [7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo(a)pyrene-(anti-isomer)] such that the only adduct is *trans*-(7R)-N^2-{10(7β,8α,9α-trihydroxy-7,8,9,10-tetrahydrobenzo[a]pyrene)-yl}-deoxyguanosine. To reiterate, a modified-DNA antiserum elicited against a highly modified immunogen DNA may not efficiently recognize adducts in DNA modified to a lower extent (Fig. 3, ELISA). This can be investigated by assaying a series of DNA samples modified to varying extents in a competitive immunoassay. In addition, the total adducts in a biological sample DNA can often be determined by an independent method, for example radiolabeling, and the results compared with those obtained by immunoassay (Table 1; Santella, 1988; van Schooten *et al.*, 1987; Santella *et al.*, 1988a, 1988b). If the difference in antibody recognition between high and low modified DNAs is in the range of 100–

1000-fold, the assay may not be useful for the quantitative monitoring of biological samples. On the other hand, a discrepancy of several-fold might be overcome by comparison of the unknown biological samples with a DNA standard modified to a similar extent (Table 1; van Schooten *et al.*, 1987; Santella *et al.*, 1988a).

An antiserum that cross-reacts with adducts or adducted DNAs formed by compounds of the same chemical class as the original immunogen may be used to estimate adducts in biological samples provided that the limitations of the data are acknowledged. For example, the BPDE-DNA antiserum (Poirier *et al.*, 1980) has been shown to cross-react with DNAs modified by a variety of polycyclic aromatic hydrocarbons (PAHs) (Weston *et al.*, 1989a). Therefore, it is likely that inhibition exhibited by human samples in the BPDE–DNA ELISA is due to the presence of a variety of adducts. Since it is not feasible to determine these adducts individually, a provisional adduct estimation has been achieved by comparison of sample %Inhibition values with a BPDE–DNA standard curve. The results have been termed "BP–DNA antigenicity" or "PAH–DNA adducts" (Liou *et al.*, 1989).

To ensure adequate reproducibility in the ELISA, triplicate experimental wells and one control well should be analyzed simultaneously on each microtiter plate. In contrast, duplicate RIA tubes generally yield consistent results. Each sample should be assayed on two separate occasions (different RIAs or different microtiter plates), and can be considered positive if the %Inhibition observed with sample DNA is on the linear portion of the standard curve.

6. INTERLABORATORY COMPARISON OF ASSAYS

Immunoassays have been used with increasing frequency for the determination of DNA adducts in human samples (Bartsch *et al.*, 1988), and it has become apparent that the results vary from laboratory to laboratory. In a collaborative study a standard assay was established in which the same reagents were used by each laboratory in order to eliminate these discrepancies (Santella *et al.*, 1988a; Fig. 3, ELISA). In retrospect, it may be unnecessary to use exactly the same reagents in order to obtain comparable results, but certain sources of variability are worthy of note.

In establishing an immunoassay, the amount of standard adduct either alone or present in a given DNA sample is generally determined by UV absorbance (Jeffrey *et al.*, 1977; Poirier, 1980), radioactivity (Santella, 1988), or atomic absorbance spectroscopy (Reed *et al.*, 1988). These determinations may vary for the same type of adduct or modified DNA, and may result in large interlaboratory discrepancies in the standard curve. At the very least, the details of each method should be documented so that reasonable comparisons can be made.

Once an assay is developed and a standard curve is established, it is important to determine whether or not all of the adducts in a biological sample are being measured. One of the easiest ways to investigate this is often to expose cultured cells to a radioactive form of the chemical in question and determine adduct formation by radiochemical analysis (Table 1; Santella *et al.*, 1988a, 1988b; Poirier, 1980). Large differences between adducts measured by immunoassay and by radioactivity may be due to several factors, which will be discussed in the following paragraphs.

As mentioned previously, if the immunogen was a highly modified DNA (e.g., 1 adduct in 10^2 bases) the antiserum may not recognize all of the adducts in a biologically-modified-DNA sample with adduct levels in the range of 1 adduct in 10^6 bases (Fig. 3, ELISA). Complete recognition has been demonstrated for DNA samples modified to high and low extents with an antiserum elicited against 8-methoxypsoralen–DNA (Santella *et al.*, 1988b) and aflatoxin–DNA (Hsieh *et al.*, 1988). However, it is possible for the discrepancy to be several-hundred-fold (Poirier *et al.*, 1988 and unpublished), in which case the antiserum may not be useful for quantitative adduct determination. If such a discrepancy is only several-fold, as in the case of anti-BPDE–DNA, accurate determination of adducts in a biological sample may be achieved by comparison of biological samples with a standard DNA modified in the same range as the samples (van Schooten *et al.*, 1987; Santella *et al.*, 1988a).

It is also possible that the problem of adduct underestimation by an immunoassay may have a completely different source, for example, the assay end point. The ELISA for measurement of BPDE–DNA has been performed with an alkaline-phosphatase-conjugated-IgG and either *p*-nitrophenyl-phosphate or methyl-umbelliferyl-phosphate as the substrate (Santella *et al.*, 1988a). The latter end point, which uses fluorescence detection, has a greater discrepancy between the measurement of the high modified standard DNA and the biological samples than the colorimetric assay, which measures *p*-nitrophenol. Accurate sample determination can be achieved, however, by using a standard modified in the range of the biological samples (Table 1). Based on these experiences, the recommendation is to test anti-modified-DNA ELISAs against a radioactively labeled biological sample to determine whether or not adduct determination is complete or underestimated with a given assay.

Underestimation of adducts may also be related to antigen presentation in the assay. For example, an anti-modified-DNA antiserum may recognize adducts on denatured DNA better than those on native DNA (Fig. 3, RIA; Poirier *et al.*, 1980), and an anti-adduct antiserum may recognize individual adducts better than denatured modified DNA and native modified DNA (Fig. 2; Poirier and Connor, 1982; Poirier, 1980).

2. COMBINATION OF IMMUNOLOGICAL AND CHEMICAL TECHNIQUES FOR DETERMINING DNA ADDUCTS IN HUMAN SAMPLES

By comparison with other techniques that measure carcinogen-DNA adducts, immunoassays have their own particular strengths and weaknesses that are based primarily on the antisera specificities. Since the antisera often recognize adducts formed by many compounds within the same chemical class (Fig. 4), and since humans are exposed to many different chemicals, it is not possible to determine specific adducts individually without further purification of the sample. In the case of the hydrocarbons, many studies have been performed in which samples have been compared to a BPDE–DNA standard, and the results termed BP–DNA antigenicity [BP denotes benzo(a)pyrene] or PAH–DNA adducts, to reflect the presence of a mixture of hydrocarbon adducts. Ethyl and methyl adducts of deoxyguanosine and deoxyadenosine have been determined by immunoassay in human DNA samples after hydrolysis and high-pressure liquid chromatography (HPLC) separation of the adducts (Umbenhauer *et al.*, 1985). Besides allowing the determination of individual adducts, a prior HPLC separation increases the assay sensitivity since adducts from virtually unlimited (mg) quantities of DNA can be assayed.

The combination of HPLC adduct purification and immunoassay is most useful for simple adduct mixtures. When mixtures of adducts, for example from PAHs (Tierney *et al.*, 1986) or aflatoxins (Groopman *et al.*, 1985),

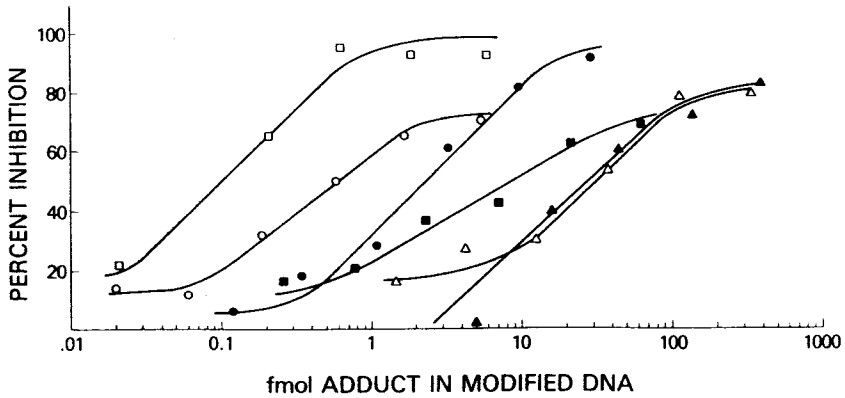

Figure 4. ELISA for BPDE–DNA (same conditions as in Fig. 3) in which the standard BPDE–DNA (1% modified) inhibitor is competed as denatured (●). The other curves are denatured DNAs, modified in the range of 0.1%–1.0%, with the diol-epoxides of chrysene (□), benzo[k]fluoroanthene (△), dibenz[a,c]anthracene (▲) and the bay region (○) and nonbay region (■) benz[a,c]anthracenes. This ELISA demonstrated that the anti-BPDE–DNA has specificity for all of the above modified DNAs.

are likely to be present in a human sample, determination of a specific adduct can be achieved by combining the antibody class specificity with other preparative and analytical techniques. Immunocolumns can be made by binding carcinogen–DNA adduct antisera to a solid matrix (Tierney *et al.*, 1986; Manchester *et al.*, 1988; Weston *et al.*, 1989a). Enzyme-hydrolyzed adducted DNA will attach to the column while unmodified DNA passes through and can later be quantitatively released (Manchester *et al.*, 1988; Weston and Poirier, unpublished). This method will serve to concentrate a specific class of adducts. The DNA samples collected in the column eluates can be subjected to further degradation, HPLC, and final identification. In order to combine immunoaffinity chromatography with highly sensitive adduct detection by ^{32}P-postlabeling (Manchester *et al.*, 1989), further method development is required for analytical specificity. Analytical techniques that could be employed at this point include fluorescence spectrophotometry (Weston *et al.*, 1989a; Manchester *et al.*, 1988), gas chromatography–mass spectrometry (Weston *et al.*, 1989b; Manchester *et al.*, 1988), or electrochemical detection (Adams *et al.*, 1986). For example, a combination of immunoaffinity chromatography and HPLC have been used to measure aflatoxin-bound guanine in the urine of Chinese individuals exposed to dietary aflatoxin (Sun *et al.*, 1988; Groopman *et al.*, 1985). In addition, a series of studies combining these techniques has been performed to characterize chemically the deoxyguanosine adduct of benzo(a)pyrene in DNA from human placenta containing a mixture of hydrocarbon adducts. Immunoassays were combined with HPLC and second-derivative synchronous scanning fluorescent spectrophotometry to obtain a chemical signal identical with the synthetic standard (Weston *et al.*, 1989a). The adducts obtained were further analyzed by ^{32}P postlabeling (Manchester *et al.*, 1989) and identified by gas chromatography–mass spectrometry (Manchester *et al.*, 1988). Although all of these steps could not feasibly be employed for routine sample testing, this type of approach is useful to assist validation of the different techniques available for human biomonitoring, and may provide essential chemical characterization of adducts found in human tissues.

8. CONCLUSIONS

Antisera specific for chemical–DNA adducts and chemically modified DNA have proven to be useful tools in the investigation of mechanisms of chemical carcinogenesis. However, the assays involved are complicated and require care; the data they yield will only be as good as the validation upon which they are established. As discussed here, the process of characterizing a new antiserum should carefully document many things, including cross-

reactivity and the extent to which adducts are recognized in a biological sample. Extension of the technology described here into the realm of human biomonitoring may provide valuable information on human exposure, human cancer epidemiology, and risk assessment.

REFERENCES

H. Bartsch, K. Hemminki, and I. K. O'Neill (eds.), *Methods for Detecting DNA Damaging Agents in Humans: Applications in Cancer Epidemiology and Prevention*, International Agency for Research on Cancer Scientific Publication No. 89, IARC, Lyon France (1988).

V. A. Bohr, C. A. Smith, D. S. Okumoto, and P. C. Hanawalt, DNA repair in an active gene: Removal of pyrimidine dimers from the DHFR gene of CHO cells is much more efficient than in the genome overall, *Cell* **40,** 359–369 (1985).

J. E. Butler, Antibody-antigen and antibody-hapten reactions. In: *Enzyme-Immunoassay*, ed. E. T. Maggio, CRC Press, Boca Raton, FL pp. 6–52 (1980).

E. Engvall, Enzyme immunoassay ELISA and EMIT, *Methods Enzymol.* **70,** 419–439 (1980).

B. F. Erlanger, The preparation of antigenic hapten-carrier conjugates: A survey, *Methods Enzymol.* **70,** 85–104 (1980).

A. M. J. Fichtinger-Schepman, R. A. Baan, A. Luiten-Schuite, M. van Kijk, and P. H. M. Lohman, Immunochemical quantitation of adducts induced in DNA by *cis*-diamminedichloroplatinum (II) and analysis of adduct-related DNA-unwinding, *Chem. Biol. Interact.* **55,** 275–288 (1985).

W. G. Flamm, M. L. Birnstiel, and P. M. B. Walker, Preparation and fractionation, and isolation of single strands of DNA by isopycnic ultracentrifugation in fixed-angle rotors. In: *Subcellular Compounds: Preparation and Fractionation*, eds. G. Birnie and S. M. Fox, Butterworth, London, pp. 129–155 (1967).

J. D. Groopman, P. R. Donahyue, J. Q. Zhu, J. S. Chen, and G. N. Wogan, Aflatoxin metabolism in humans: detection of metabolites and nucleic acid adducts in urine by affinity chromatography, *Proc. Natl. Acad. Sci USA* **82,** 6492–6496 (1985).

J. D. Groopman, L. J. Trudel, P. R. Donahue, A. Marshak-Rothstein, and G. N. Wogan, High-affinity monoclonal antibodies for aflatoxins and their application to solid-phase immunoassays, *Proc. Natl. Acad. Sci. USA* **81,** 7728–7731 (1984).

C. C. Harris, R. H. Yolken, and I.-C. Hsu, Enzyme immunoassays: Applications in cancer research, *Methods Cancer Res.* **20,** 213–243 (1982).

P. J. Hertzog, A. Shaw, J. R. Lindsay Smith, and R. C. Garner, Improved conditions for the production of monoclonal antibodies to carcinogen-modified DNA, for use in enzyme-linked immunosorbent assays (ELISA), *J. Immunol. Methods* **62,** 49–58 (1983).

L.-L. Hsieh, S.-W. Hsu, D.-S. Chen, and R. M. Santella, Immunological detection of aflatoxin B_1-DNA adducts formed *in vivo*, *Cancer Res.* **48,** 6328–6331 (1988).

A. M. Jeffrey, I. B. Weinstein, K. W. Jennette, K. Grzeskowiak, K. Nakanishi, R. G. Harvey, H. Autrup, and C. Harris, Structures of benzo(a)pyrene-nucleic acid adducts formed in human and bovine bronchial explants, *Nature* **269,** 348–349 (1977).

D. S. Kabakoff, Chemical aspects of enzyme-immunoassay. In: *Enzyme-Immunoassay*, ed. E. T. Maggio, CRC Press, Boca Raton, FL, pp. 71–104 (1980).

J. L. Klotz, Enzyme-linked immunosorbent assay for antibodies to native and denatured DNA, *Methods Enzymol.* **84,** 194–201 (1982).

E. Kriek, L. Den Engelse, E. Scherer, and J. G. Westra, Formation of DNA modifications by chemical carcinogens identification, localization and quantification, *Biochim. Biophys. Acta* **738,** 181–201 (1984).

M. Leng, Immunological detection of lesions in DNA, *Biochim.* **67,** 309–315 (1985).

S.-H. Liou, D. Jacobson-Kram, M. C. Poirier, D. Nguyen, P. T. Strickland, and M. S. Tockman, Biological monitoring of firefighters: Sister chromatid exchange and polycyclic aromatic hydrocarbon-DNA adducts in peripheral blood cells, *Cancer Res.*, **49,** 4929–4935 (1989).

S. J. Lippard, H. M. Ushay, C. M. Merkel, and M. C. Poirier, Use of antibodies to probe the stereochemistry of antitumor platinum drug binding to deoxyribonucleic acid, *Biochem.* **22,** 5165–5168 (1983).

D. K. Manchester, A. Weston, J.-S. Choi, G. E. Trivers, P. V. Fennessey, E. Quintana, P. B. Farmer, D. L. Mann, and C. C. Harris, Detection of benzo[a]pyrene diol epoxide-DNA adducts in human placenta, *Proc. Natl. Acad. Sci. USA* **85,** 9243–9247 (1988).

D. K. Manchester, V. L. Wilson, I. C. Hsu, J. S. Choi, N. B. Parker, D. L. Mann, A. Weston, and C. C. Harris, Synchronous fluorescent spectroscopic, immunoaffinity chromatographic and ^{32}P-postlabeling analysis of human placental DNA known to contain benzo[a]pyrene diol epoxide adducts, *Carcinogenesis*, **11,** 553–559, 1990.

R. J. Mayer and J. H. Walker, Uses of antisera. In: *Immunochemical Methods in the Biological Sciences: Enzymes and Proteins*, Academic Press, New York (1980).

S. A. Miller, D. D. Dykes, and H. F. Polesky, A simple salting out procedure for extracting DNA from human nucleated cells, *Nucleic Acids Res.* **16,** 1215 (1988).

R. Müller and M. F. Rajewsky, Antibodies specific for DNA components structurally modified by chemical carcinogens, *J. Cancer Res. Clin. Oncol.* **102,** 99–113 (1981).

T. W. Munns and M. K. Liszewski, Antibodies specific for modified nucleosides: An immunochemical approach for the isolation and characterization of nucleic acids, *Prog. Nucleic Acid Res. Mol. Biol.* **24,** 109–165 (1980).

J. Adams, M. David and R. W. Giese, Pentafluorobenzylation of O^4-Ethylthymidine and Analogues by Phase Transfer catalysis for determination by Gas Chromatography with electron capture detection, *Anal. Chem.*, **58,** 345–348, (1986).

F. P. Perera, M. C. Poirier, S. H. Yuspa, J. Nakayama, A. Jaretzki, M. M. Curnen, D. M. Knowles, and I. B. Weinstein, A pilot project in molecular cancer epidemiology: Determination of benzo[a]pyrene-DNA adducts in animal and human tissues by immunoassays, *Carcinog.* **3,** 1405–1410 (1982).

D. H. Phillips, Modern methods of DNA adduct determination. In: *Handbook of Experimental Pharmacology*, Vol. 94/I eds. P. L. Glover and C. S. Cooper, Springer-Verlag, Berlin (1989), pp. 503–546.

O. J. Plescia and W. Braun, Nucleic acids as antigens, *Adv. Immunol.* **6,** 231–252 (1967).

A. C. M. Plooy, A. M. J. Fichtinger-Schepman, H. H. Schutte, M. van Kijk, and P. H. M. Lohman, The quantitative detection of various Pt-DNA-adducts in Chinese hamster ovary cells treated with cisplatin: application of immunochemical techniques, *Carcinog.* **6,** 561–566 (1985).

M. C. Poirier, Measurement of formation and removal of adducts of N-acetoxy-2-acetylaminofluorene. In: *DNA Repair,* Vol. 1, Part A, eds. E. C. Friedberg and P. C. Hanawalt, Dekker, New York, pp. 143–153 (1980).

M. C. Poirier, Antibodies to carcinogen-DNA adducts, *J. Natl. Cancer Inst.* **67,** 515–519 (1981).

M. C. Poirier, The use of carcinogen-DNA adduct antisera for quantitation and localization of genomic damage in animal models and the human population, *Environ. Mutagen.* **6,** 879–887 (1982).

M. C. Poirier, and R. J. Connor, Radioimmunoassay for 2-acetylaminofluorene-DNA adducts, *Methods Enzymol.* **84,** 607–618 (1982).

M. C. Poirier, M. J. Egorin, A. M. J. Fichtinger-Schepman, and E. Reed, DNA adducts of cisplatin and carboplatin in tissues of human cancer patients. in: *Methods for Detecting Damaging Agents in Humans: Applications in Cancer Epidemiology and Prevention,* eds. H. Bartsch, K. Hemminki, and I. K. O'Neill, International Agency for Research on Cancer Scientific Publication No. 89, Lyon, France, pp. 313–320 (1988).

M. C. Poirier, R. Santella, I. B. Weinstein, D. Grunberger, and S. H. Yuspa, Quantitation of benzo(a)pyrene-deoxyguanosine adducts by radioimmunoassay, *Cancer Res.* **40,** 412–416 (1980).

M. C. Poirier, S. H. Yuspa, I. B. Weinstein, and S. Blobstein, Detection of carcinogen-DNA adducts by radioimmunoassay, *Nature* **270,** 186–188 (1977).

M. F. Rajewsky, R. Müller, J. Adamkiewicz, and W. Drosdzick, Immunological detection and quantification of DNA components structurally modified by alkylating carcinogens (ethylnitrosourea). In: *Carcinogenesis: Fundamental Mechanisms and Environmental Effects,* eds. B. Pullman, P. O. P. Ts'o, and H. Gelboin, Reidel, Dordrecht, pp. 207–218 (1980).

E. Reed, S. Sauerhoff, and M. C. Poirier, Quantitation of platinum-DNA binding after therapeutic levels of drug exposure—a novel use of graphite furnace spectrometry, *At. Spectrosc.* **9,** 93–95 (1988).

E. Reed, S. Gupta-Burt, C. L. Litterst and M. C. Poirier, Characterization of the DNA damage recognized by an antiserum elicited against *cis*-diammine-dichloroplatinum (II)-Modified DNA, *Carcinogenesis,* in press, 1991.

D. W. Roberts, R. W. Benson, T. J. Flammang, and F. F. Kadlubar, Development of an avidin-biotin amplified enzyme-linked immunoassay for detection of DNA adducts of the human bladder carcinogen 4-aminobiphenyl. In: *Mechanisms of DNA Damage and Repair: Implications for Carcinogenesis and Risk Assessment,* eds. M. G. Simic, L. Grossman, and A. D. Upton, Plenum Press, New York, pp. 479–488 (1986).

R. M. Santella, Application of new techniques for the detection of carcinogen adducts to human population monitoring, *Mutat. Res.* **205,** 271–282 (1988).

R. M. Santella, A. Watson, F. P. Perara, G. T. Trivers, C. C. Harris, T. L. Young, D. Nguyen, B. M. Lee, and M. C. Poirer, Interlaboratory comparison of antisera and immunoassays for benzor[a]pyrene-diol-epoxide-I-Modified DNA, *Carcinog.* **9,** 1265–1269 (1988a).

R. M. Santella, X. Y. Yang, V. A. DeLeo, and F. P. Gasparro, Detection and quantification of 8-methoxypsoralen-DNA adducts. In: *Methods for Detecting DNA Damaging Agents in Humans: Applications in Cancer Epidemiology and Prevention,* International Agency for Research on Cancer Scientific Publication No. 89, eds. H. Bartsch, K. Hemminki, and I. K. O'Neill, IARC, Lyon, France, pp. 333–340 (1988b).

D. L. Sawicki, S. M. Beiser, D. Srinivasan, and P. R. Srinivasan, Immunochemical detection of 7-methylguanine residues in nucleic acids, *Arch. Biochem. Biophys.* **176,** 457–464 (1976).

B. D. Stollar, The experimental induction of antibodies to nucleic acids, *Methods Enzymol.* **70,** 70–85 (1980).

P. T. Strickland and J. M. Boyle, Immunoassay of carcinogen-modified DNA, *Prog. Nucleic Acid Res. Mol. Biol.* **31,** 1–58 (1984).

B. Tierney, A. Benson, and R. C. Garner, Immunoaffinity chromatography of carcinogen DNA adducts with polyclonal antibodies directed against benzo[a]pyrene diol-epoxide-DNA, *J. Natl. Cancer Inst.* **77,** 261–267 (1986).

G. E. Trivers, C. C. Harris, C. Rougeot, and F. Dray, Development and use of ultrasensitive enzyme immunoassays, *Methods Enzymol.* **103,** 409–434 (1983).

D. Umbenhauer, C. P. Wild, R. Montesano, R. Saffhill, J. M. Boyle, N. Huh, U. Kirstein, J. Thomale, M. F. Rajewsky, and S. H. Lu, O^6-Methyldeoxyguanosine in oesophageal DNA among individuals at high risk of oesophageal cancer, *Int. J. Cancer* **36,** 661–665 (1985).

F. J. van Schooten, E. Kriek, M. S. T. Steenwinkel, H. P. J. M. Noteborn, M. J. X. Hillebrand, and F. E. V. Leeuwen, The binding efficiency of polyclonal and monoclonal antibodies to DNA modified with benzo[a]pyrene diol epoxide is dependent on the level of modification. Implications for quantitation of benzo[a]pyrene-DNA adducts *in vivo, Carcinog.* **8,** 1263–1269 (1987).

A. Weston, D. K. Manchester, M. C. Poirier, J.-S. Choi, G. E. Trivers, D. L. Mann, and C. C. Harris, Derivative fluorescence spectral analysis of polycyclic aromatic hydrocarbon-DNA adducts in human placenta, *Chem. Res. Toxicol.,* **2,** 104–108, (1989).

A. Weston, D. K. Manchester, A. Povey, and C. C. Harris, Detection of carcinogen-macromolecular adducts in humans, *J. Am. Coll. Physicians,* **8,** 913–932, (1989).

G. B. Wisdom, Enzyme-immunoassay, *Clin. Chem.* **22,** 1243–1255 (1976).

A. Zettner, Principles of competitive binding assays (saturation analyses). I. Equilibrium techniques, *Clin. Chem.* **19,** 699–705 (1973).

A. Zettner and P. E. Duly, Principles of competitive binding assays (saturation analyses). II. Sequential saturation, *Clin. Chem.* **20,** 5–14 (1974).