Immunological Methods for Detection of Carcinogen-DNA Damage in Humans

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Introduction
The demonstration in the mid-1960s and early 1970s that antibodies could be generated against the normal nucleosides suggested that antibodies to modified nucleosides were also possible (1). The first antiserum against modified DNA recognized the alkylated adducts of several bases including O6-methyl and O6-ethylguanine, 7-methylguanine, and N4-methyl-adenine (reviewed in Ref. 2). For bulky chemical carcinogens, the first antibodies were against the adducts of N2-acetylaminofluorene (3, 4). Because humans are not exposed to this agent, these antisera have been limited to studies on cultured cells or treated animals. However, this work opened up a new area of research and was soon followed by antibodies recognizing adducts formed by BPDE (5, 6). As described below, the use of immunological methods for monitoring DNA damage in humans is well established. The goal of this review is to introduce the methods of antibody development and their application to adduct detection. Although there have been many reports of antibody development and use in in vitro studies, data reviewed below is generally limited to studies of adduct detection in humans. This area has expanded rapidly over the past 10 years, and although an effort has been made to include representative studies for all types of adducts, the list may be incomplete.

Methods
Antibody Development. Two types of antigens have been used for the development of antibodies to carcinogen-damaged DNA, intact modified DNA electrostatically complexed to methylated carrier protein and monooxidated coupled to carrier protein. With DNA antigens, high modification levels, generally near one modified base in 100 nucleotides, are required for the successful generation of titer. Thus, highly reactive intermediates of the carcinogen of interest must be available for reaction in vitro with DNA. With monoadduct antibodies, coupling to a carrier protein such as keyhole limpet hemocyanin is necessary. There are numerous methods for coupling of modified bases, nucleosides, and nucleotides to proteins, and these methods and the use of adjuvants have been described previously (5, 7). The Cold Spring Harbor monographs, Antibodies: A Laboratory Manual (8) and Using Antibodies (9), are excellent sources for detailed information on all aspects of antibody development, purification, and use.

The choice of antigen is dictated by its ease of synthesis and the ultimate use of the antibody. For example, if the ultimate goal is immunoaffinity purification of the modified nucleoside from DNA digests, a monoadduct antigen is preferred. A lower affinity antibody might also be preferable to increase the ease of recovery of antigen from the immunoaffinity column. If the goal is immunohistochemical detection of adducts in intact cells, a modified DNA antigen is generally preferable, but a high affinity antibody is essential.

After the antibody is generated and characterized in terms of sensitivity of recognition of the original antigen, it is necessary to test for cross-reactivity to other adducts that may be structurally similar. Such cross-reactivity occurs frequently and may result in difficulties in quantification of a specific antigen in certain types of samples containing multiple adducts. For example, the first polyclonal antiserum generated against BPDE-DNA was found to be highly specific for this adduct and did not recognize adducts resulting from several other classes of chemical carcinogens (6). However, when diol epoxides of other PAHs were synthesized and used to modify DNA, cross-reactivity was observed with both the polyclonal and monoclonal anti-BPDE-DNA antibodies (10, 11). Humans are exposed to BP as part of a complex mixture of multiple PAHs that may form diol epoxides, bind to DNA, and be recognized by the antibody, but with different affinities. Thus, absolute adduct levels cannot be determined because these multiple adducts are likely to be present in human samples. However, measurement of the class of adducts as a whole is still a useful marker. Cross-reactivity was also observed with antibodies recognizing alkylated DNA adducts, but because these antibodies recognize monoadducts, specificity can be increased by digestion of DNA and chromatography before immunoassay analysis (2).

Both polyclonal and monoclonal antibodies have been developed against carcinogen-DNA adducts. Polyclonal antisera are quicker to generate, requiring only the immunization of animals several times over the course of 2–3 months, followed by collection of sera. For monoclonal antibody development, immunization is followed by the hybridization of spleen cells from animals showing titer with myeloma cells of an appropriate genetic strain. Hybrid clones must be cultured and tested for specificity, then subcloned. This can add an additional 3–4 months to the process. The major advantage of monoclonals is the unlimited supply of antibody that is available by growing the cells as ascites tumors in animals or by the presently more preferable in vitro hollow fiber cell culture technique. In general, similar sensitivities and specificities can be obtained with either polyclonal or monoclonal antibodies.
After antibodies have been developed and characterized, they can be used in a wide variety of assays, as outlined below. However, before use on biological samples, it is important to carry out experiments to validate the particular assay to be used. Laboratory validation should ideally use an alternate method of quantification of the DNA modification level. For example, the immunoassay for quantification of damage levels in animals treated with $[^{3}H]$BP indicated similar modification levels by immunoassay and radiolabel counting if the standard BPDE-DNA used in the assay was modified in a similar range as the biological samples (i.e., 1 adduct/10$^6$ nucleotides; Ref. 6). An immunofluorescence method for detection of 4-aminobiphenyl-DNA was validated by analysis of DNA from treated animals by GC/MS methods (12). These studies demonstrated the reliability of the assays at high doses and thus relatively high adduct levels. However, it is now known that some antibodies generated against highly modified DNA bind adducts with better affinity in samples with high modification levels compared with the much lower modification levels found in human samples (reviewed in Ref. 13). This has resulted in the recognition that the modification level of the DNA used in the standard curve should be similar to that in the samples to be tested. For these reasons, it would obviously be preferable to carry out assay validation studies using human samples that have lower levels of multiple carcinogen-DNA adducts rather than using samples from animals treated with high doses of a single chemical. However, alternate methods of quantification may not be available for these low levels or, if available, they may also have uncertainties in terms of accuracy in quantitation. For example, a comparison of 4-ABP-DNA adduct levels in a sample modified in vitro to a low level (62 ± 0.8 adducts/10$^6$ nucleotides), based on $^{3}H$ incorporation from labeled carcinogen, found that determination of modification level by postlabeling, GC/MS, and immunoassay resulted in values ranging from 0.84 (postlabeling) to 82 (immunoassay)/10$^6$ (14).

There are very few studies that have correlated adducts in human tissues detected by immunoassay with those determined by postlabeling (15, 16) or GC/MS (17). Although correlations have been reasonable, differences in absolute adduct levels have been observed.

**RIA.** Anti-carcinogen adduct or carcinogen modified-DNA antibodies were first used in RIA in which the antigen is synthesized in both a radiolabeled (tracer) and nonlabeled (inhibitor) form (3, 18). Standard curves are generated by mixing fixed amounts of antibody and tracer with increasing concentrations of inhibitor in a constant volume. With larger amounts of inhibitor, less radioactive material is bound by the antibody. Primary antibody is then precipitated, usually with a secondary antiserum, and radioactivity in the pellet or supernatant is counted. Unknowns are similarly mixed with antibody and tracer, and antigen concentration is determined with the standard curve. Although highly sensitive and reproducible, RIAs have generally been replaced by ELISAs, which do not require the use of radioactive material and the associated handling problems. In ELISA, the RIA tracer is replaced by a constant amount of immunogen bound to a microtiter plate.

**Competitive ELISAs.** Competitive ELISA is the most commonly used immunological method for quantification of DNA damage. There are numerous variations of the ELISA using alkaline phosphatase or peroxidase-conjugated secondary antisera for primary antibody detection and colored, fluorescent, or radioactive substrates for end point detection. Antigen, either in vitro modified DNA or monoadduct coupled to carrier protein, is coated onto 96-microwell plates, and nonspecific binding to the plate is blocked by incubation of the wells with a dilute protein solution. A standard curve is generated by serial dilution of either the modified denatured DNA or monoadduct and mixed with diluted antibody. Unknowns to be tested are similarly mixed with antibody before addition to the plate. Antibody binding to the antigen on the plate is competed by antigen in solution. After incubation and washing off nonbound material, bound primary antibody is quantitated with enzyme-conjugated secondary antiserum, followed by the appropriate substrate. With the highest affinity antibodies, 50% inhibitions can be obtained with adduct levels in the femtomol range. With this level of sensitivity and the ability to assay 50–100 μg of DNA, adduct levels of ~1 adduct/10$^6$ nucleotides can be measured. In assays in which the monoadduct is isolated before analysis, sensitivity can be further increased provided larger amounts of DNA are available.

**Immunohistochemistry.** Immunohistochemical detection of DNA damage can be carried out on either fixed cells (e.g., lymphocytes or exfoliated oral or bladder cells) or tissue sections (frozen or paraffin). Although some studies directly incubate fixed material with primary antibody, procedures to enhance antibody binding are usually carried out to increase assay sensitivity. These procedures normally include treatment with proteases to remove histone and nonhistone proteins from the DNA and with RNase to eliminate potential cross-reactivity with RNA adducts. This can be followed by acid or base treatment to denature the DNA and further increase antibody accessibility to the adduct. Clearly the adduct of interest must be stable to the denaturing treatment chosen. There are two commonly used detection systems for visualization of bound, adduct-specific antibodies, immunofluorescence or immunoperoxidase. Cells can be counterstained with propidium iodide or ethyl green, for immunofluorescence or immunoperoxidase staining, respectively, to allow visualization of nuclei in adduct-negative cells.

Samples can be evaluated in a qualitative or semiquantitative manner. Qualitative assessment uses subjective estimation of staining intensity and/or number of positively stained cells with an arbitrary scale. Quantitative measurements for both fluorescence and peroxidase staining can directly measure intensity of either fluorescence or absorption of the nucleus, respectively, using sophisticated video cameras and software.

For immunohistochemical assays, it is important to run control experiments to demonstrate the specificity of cell staining. These controls should include cells treated in culture or tissues from animals treated in vivo with or without the chemical of interest. Additional controls routinely include samples containing adducts but treated with DNase before incubation with primary adduct-specific antibody or with primary antibody that has been preabsorbed with the DNA adduct of interest. Nonspecific antibodies are also frequently used to confirm specificity of staining.

The major advantages of the immunohistochemical method are its ability to detect adducts in specific cell types within a tissue and its applicability to small amounts of sample. Less than one ml of blood is required for analysis of lymphocytes compared with the 30 ml required for isolation of sufficient amounts of DNA for ELISA. As examples below illustrate, it is also applicable to stored paraffin sections, widely expanding the types of studies that can be carried out. However, care must be taken to ensure that the samples were handled uniformly to avoid artifacts due to sample processing. The disadvantages of the immunohistochemical method are those of all immunoassays. Cross-reacting antibodies will result in er-
rors in quantification, and in general, the method is not as sensitive as the competitive ELISA.

**Immunofluorescence Purification.** Antibodies can also be used for the purification of adducts from digested DNA or other biological material such as urine. Immunofluorescence chromatography provides a rapid and efficient means for sample enrichment and can be followed by ELISA, HPLC, 32P postlabeling, or GC/MS analysis. For example, the method has been used to analyze 3-methyladenine in urine (2). Isolation of the modified base by immunofluorescence chromatography was followed by quantification with ELISA, GC/MS, or RIA. This method was also used to prove that BP-deoxyguanosine adducts are present in placental and lung tissue (19, 20).

**Other Approaches.** Slot or dot blot methods have also been developed for detection of adducts in DNAs immobilized on nitrocellulose. Incubation with primary antibody is followed by washing then incubation with enzyme-labeled secondary anti-serum. Substrates producing colored or chemiluminescent products have been used. An assay for PAH-DNA reported a sensitivity of 2/106 with a colored end point (21). Malondialdehyde-DNA adducts were detected with a limit of sensitivity of 2.5/106 using peroxidase-conjugated secondary antisera and a chemiluminescence end point (22). These methods have not yet been used extensively on human samples.

An ultrasensitive assay for measuring DNA damage was described recently that couples immunochromatography with capillary electrophoresis and laser-induced fluorescence detection (23). A detection limit for thymine glycols of 3/1021 was reported, but this was with DNA modified in vitro.

Flow cytometric methods have also been applied to detection of adducts. We used it to investigate differences in in vitro DNA adduct formation by 8-MOP during the cell cycle (24). Another report applied the method to detection of BP adducts in human lymphocytes treated in vitro with the carcinogen (25). It is not clear that the method is sufficiently sensitive for detection of adducts formed in vivo.

**Measurement of DNA Damage in Humans**

PAHs. We and others have developed polyclonal and monoclonal antibodies recognizing PAH-DNA adducts from animals immunized with BPDE-I-modified DNA (reviewed in Ref. 13). Initial studies referred to quantification of BPDE adducts; but with the recognition of antibody cross-reactivity, described above, studies in humans now indicate the presence of PAH-DNA adducts or BPDE-DNA antigenicity. Because blood is readily available from healthy subjects, it has been used extensively as a surrogate for tissue adduct levels. Initial studies measured damage in total WBC DNA, but more recent studies have used DNA from the longer lived mononuclear cell fraction. There are few reports on the correlation between target and surrogate PAH-DNA adduct levels in humans. Correlations between lung and total white blood adduct levels by immunohistochemical staining techniques for monitoring PAH-DNA (52, 55), the first study of human tissues demonstrated adducts in bronchial cells, cervical epithelium, oocytes, and placental tissue (54). Adducts were also found in bronchial cells in another early report (53). A recent report on 3–8-cell stage embryos of patients undergoing in vitro fertilization found higher levels of adducts in embryos in which either partner smoked (56).

Our own initial studies were on skin biopsies of coal tar-treated psoriasis patients (57). Elevated levels of fluorescence staining, indicating increased adduct formation, were found in epidermal cells of patients compared with controls. Lymphocytes from individuals working in a Polish coke oven factory, living near it, or in a rural region were analyzed using a quantitative fluorescence method (58). In agreement with prior ELISA data, adducts were significantly higher in both the occupational and environmental groups compared with the rural control group (15, 37). The immunofluorescence method was also applied to exfoliated oral cells of smokers and non-smokers. However, auto-fluorescence of unstained oral cells necessitated the use of the immunoperoxidase method (59, 60). Quantification of peroxidase staining indicated about a 2-fold increase in PAH-DNA in smokers compared with nonsmokers, and similar data were obtained on exfoliated bladder cells of smokers and nonsmokers (61). The peroxidase method was also applied to the detection of damage in human arterial tissue using internal mammary arteries collected during coronary by-
pass surgery (62). Smoking-related staining was observed in both endothelial and smooth muscle cells.

**Aflatoxin B1.** Several antibodies have been developed for detection of exposure to the dietary mold contaminant, aflatoxin B1, to investigate its role in liver cancer etiology in conjunction with HBV infection (reviewed in Ref. 13). We generated antibody 6A10 from animals immunized with DNA containing the imidazole ring-opened form of the guanine adduct and used immunofluorescence analysis to demonstrate that ~30% of liver tissues collected at the time of surgery from HCC patients from Taiwan had detectable adducts (63). Smearred liver tissue obtained at the time of biopsy for diagnosis had a higher detection rate of 70% (64). More recently, we tested tissues from HCC cases as well as surgical controls (65). Both chronic HBV carrier status and liver AFB1-DNA adducts were significantly higher in cases than in controls, with odds ratios of 8.4 and 5.2, respectively. Moreover, HCC risk was greatest in individuals with both AFB1-DNA adducts and HBV, suggesting a viral-chemical interaction. AFB1-DNA adducts were also associated with p53 protein and DNA mutations but with borderline significance.

Immunoaffinity columns recognizing aflatoxin metabolites and excised DNA adducts have been used for their isolation from urine of subjects living in Shanghai, China in a nested case-control study followed by HPLC quantification (66). Aflatoxin metabolites or DNA adducts were found more frequently in urine collected from healthy individuals who went on to develop liver cancer than in those who did not. A synergistic interaction with virus was observed. We have confirmed these data in a Taiwanese population using both aflatoxin metabolites in urine and aflatoxin-albumin adducts in blood as a surrogate for DNA adducts (67).

**4-ABP.** 4-ABP, an aromatic amine present in cigarette smoke, is an established animal carcinogen, and in humans, has been associated with urinary bladder cancer. It is metabolized in vivo to a reactive N-hydroxy arylamine that binds covalently to DNA; the major adduct results from binding at the C8 position of guanine. Antibodies recognizing 4-ABP-DNA have been developed with both monoadduct (68) and modified (12) DNA antigens. The monoadduct antisera was used to detect adducts in digested lung and bladder DNA (69, 70).

Our method for quantitative immunofluorescence analysis of adducts (12) was validated by comparison of adduct levels measured in 4-ABP-treated mouse liver to those determined by alkaline hydrolysis release of 4-ABP from DNA isolated from the tissue, followed by GC with negative ion chemical ionization MS. A good correlation ($r = 0.98$, $P < 0.0001$) was found between relative fluorescence intensity and adduct levels determined by GC/MS. On the basis of the adduct levels determined by GC/MS, the immunohistochemical method has a limit of sensitivity of approximately 1 adduct/107 nucleotides.

The immunoperoxidase method was then used for detection of adducts in exfoliated oral (61, 71) and bladder (61) cells of smokers and nonsmokers. Higher adducts were observed in smokers than in nonsmokers. Studies of stored paraffin blocks of bladder tumor tissue found dose (number of cigarettes smoked/day)-related increases in 4-ABP-DNA and an association with mutant p53 protein expression (72). Laryngeal tissues (tumor, polyp, and normal) also demonstrated smoking-related adducts (73). The same liver tissues from HCC cases and controls that were analyzed for AFB1-DNA adducts, discussed above, were also analyzed for 4-ABP-DNA; higher levels of damage were observed in cases compared with controls (74).

**Oxidative Damage.** 8-OHdG is recognized as a useful marker for the estimation of DNA damage produced by oxygen radicals generated endogenously or exogenously. Although numerous oxidative lesions occur in DNA, oxidation of the C8 of guanine is one of the more abundant types and is also a major mutagenic lesion producing predominately G→T transversion mutations. We and others have developed antibodies for quantification of 8-OHdG in human samples and extraction of the damaged base from urine (75–78). Our antibody 1F7 was used for immunoaffinity isolation of 8-OHdG from DNA hydrolysates, followed by ELISA quantification with 1F11 (78). To validate the assay, DNA extracted from human placental tissue was assayed by ELISA and high performance liquid chromatography with electrochemical detection. Values by both methods correlated well, but the ELISA values were higher than those determined by HPLC.

We applied the immunoperoxidase method with antibody 1F7 to oral mucosal cells from smokers and nonsmokers (79). Higher levels of specific nuclear staining were observed in every smoker compared with their matched nonsmoker. The mean level of relative staining was elevated almost 2-fold in smokers compared with nonsmokers, but there was no association between staining intensity and the number of cigarettes smoked/day. The method was used most recently on nasal biopsies from children living in Mexico City, who are exposed to high levels of air pollutants, and from children living in a much less polluted coastal town (80). Higher levels (2–3-fold) of oxidative damage were observed in exposed compared with control children. We are presently using the method to determine whether oxidative damage can be influenced by an antioxidant vitamin intervention.

**Alkylation Damage.** One of the first biomonitoring papers measured O6-methyldeoxyguanosine in esophageal DNA of Chinese at high risk of esophageal cancer and of lower risk Europeans (18). The method involved hydrolysis of the DNA and isolation of adduct by HPLC before quantification by RIA. Higher adduct levels were found in the samples from China compared with those from Europe. In a later small study of placental tissues, no relationship between smoking history and O6-methyldeoxyguanosine was observed (81). Several other alkylated adducts including 7-methyldeoxyguanosine and O6-methylthymidine have also been detected (reviewed in Ref. 2). Depending on the tissue studied, differences in alkylations levels in tumor and nontumor tissue were found (82–84).

**UV Light.** One of the first immunohistochemical studies measured UV damage and repair in human skin exposed in vivo (85). Pyrimidine dimers were detected after a 0.5 minimal erythemal dose. At higher doses, staining was observed throughout the epidermis and dermis. Later studies have followed formation of both thymine dimers and 6-4 photo products and their removal (86, 87). Repair of the 6-4 photo product was faster (half-life, 2.3 h) than that of thymine dimers (half-life, 33 h; Ref. 87).

**Chemotherapeutic Agents.** In addition to the topical coal tar treatment described above, psoriasis patients are treated p.o. with the photoactivated drug 8-MOP, followed by skin irradiation with UVA. This treatment results in the formation of 8-MOP-DNA adducts in the skin, which are readily detected by immunofluorescence methods (88). In contrast, no adducts could be detected in lymphocytes isolated from treated patients (89).

\textit{cis}-Diaminedichloroplatinum(II)-DNA damage has been measured with two types of antibodies, those against modified DNA and against the individual adducts (reviewed in...
Ref. 13). Digestion of DNA and chromatographic separation of adducts before analysis is required for accurate quantification of the individual adducts, whereas with the anti-DNA antibodies, only a fraction of the adducts are detected. An immunohistochemical method quantitated damage in buccal and urinary cells of treated patients (90). Consecutive daily treatments resulted in damage proportional to the cumulative dose of cis-diaminedichloroplatinum(II). Adducts have also been monitored in tumors after treatment (91). More importantly, several of these studies demonstrated a relationship between response to therapy and formation of DNA adducts, suggesting that the method could be useful in determining candidates for chemotherapy.

Acrolein-DNA adducts were measured in leukocyte DNA of cyclophosphamide-treated cancer patients by both ELISA or immuno dot blot (92). Using an ELISA, an ~72-h half life was determined for N\(^2\)-methylguanine in dacarbazine-treated cancer patients (93).

AZT is being used extensively to treat adults and pregnant women infected with human immunodeficiency virus-1. Antibodies to AZT were used recently to monitor levels of incorporation into DNA of mononuclear cells from treated adults and newborns. Detectable levels were found in 76% of the subjects, with some samples having as many as four AZT per 10\(^4\) nucleotides (94).

Summary

Antibodies have been developed against a wide array of carcinogen-DNA adducts as well as UV-damaged or oxidized bases. Their sensitivity for the detection of DNA damage in humans has been demonstrated in numerous studies of occupational, dietary, environmental, clinical, and lifestyle exposures. Unlike the postlabeling assay that can detect multiple hydrophobic carcinogen adducts in a single experiment, specific antibodies must be developed to each adduct or class of adducts of interest. Although we have routinely distributed our monoclonal antibodies to other researchers, these antibodies and those developed by others are now commercially available, making this method of adduct detection readily accessible. Although the data generated may not be as absolutely quantitative as that obtained in other types of assays, the adduct levels found in different populations provide important information on exposure monitoring and should help in the understanding of individual cancer risk. The ease with which samples can be assayed also makes immunoassays appropriate for large scale molecular epidemiological studies. Finally, their applicability to paraffin sections permits the analysis of adducts in samples that are unsuitable for analysis by other DNA adduct measurement methods. As evidenced by the data summarized here, the immunological approach to human biomonitoring is well established and has an important role to play in studies to understand cancer etiology and prevention.

References


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