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Use of Monoclonal and Polyclonal Antibodies Against DNA Adducts for the Detection of DNA Lesions in Isolated DNA and in Single Cells

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Interaction of genotoxic chemicals with their intracellular target, i.e., DNA, may result in the formation of covalent adducts. Various methods have been developed to estimate exposure to genotoxic chemicals by means of molecular dosimetry of DNA adducts. Such experiments have generally been carried out with radiolabeled genotoxicants administered *in vitro* to cultured cells or *in vivo* to laboratory animals. Biomonitoring of human exposure to genotoxic chemicals requires methods to detect very small quantities of nonradioactive DNA adducts in limited amounts of sample.

Attention has been devoted to the development of immunochemical techniques in which specific DNA adducts can be detected with antibodies. The level of sensitivity achieved in these experiments renders these methods applicable for human biomonitoring. When suitable antibodies are available, the immunochemical approach enables one to analyze various types of adducts separately, and to discriminate between irrelevant (e.g., quickly repairable) and relevant lesions (key lesions) with respect to biological end points such as mutation induction and cancer.

Polyclonal and monoclonal antibodies were used for the detection of DNA adducts in animal and human tissue. Adducts were measured in DNA from various organs of rats treated with the liver carcinogen 2-AAF. Human exposure to genotoxic agents was studied by the measurement of DNA adducts in blood cells from patients treated with the genotoxic cytostatic cisplatin. Also, the development is described of a system to detect and quantitate DNA adducts at the single-cell level by means of immunofluorescence microscopy, which allows the analysis of small samples of human tissue with preservation of cell morphology.

Introduction

In chemical carcinogenesis, interaction of a reactive chemical agent with DNA—resulting in the formation of a covalent adduct—has been recognized as an early and possibly crucial event during the onset of the carcinogenic process (*1,2*). Demonstration of adduct formation is direct proof that interaction between the genotoxic chemical, often after metabolic conversion into a reactive metabolite (*3*), and its intracellular target has occurred. The total amount of DNA adducts can be regarded as a direct measure of the dose to which the target has been exposed. This target dose may differ considerably from the exogenous dose, i.e., the concentration of the agent to which the organism as a whole has been exposed, because of the various factors that modulate the extent of adduct formation, such as metabolic activation or detoxification, accumulation, or excretion (*4,5*). The balance between these processes may be quite different between organs and species (*6*). In the human situation, exposure may result from the occurrence of carcinogenic agents at the workplace or in the general environment. Furthermore, the effects may be strongly influenced by highly variable factors such as diet, smoking habits and the use of medication (*7–9*). Measurement of the target dose will therefore provide a more accurate estimate of biologically effective exposure (*10*).

Once the DNA damage has been inflicted, the action of repair enzymes and their effectiveness will determine whether or not a lesion will persist and hence become potentially harmful (*11*). It is generally believed that mutations in DNA mainly originate from damage that is left unrepaired or is repaired in an erroneous way. Mutations in somatic cells are thought to represent an early stage in carcinogenesis (*12,13*), whereas mutations

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in germ cells may give rise to heritable diseases, among which there are some tumors of a hereditary nature (*14*). In several cases, the extent of formation of DNA adducts has been correlated in animal experiments with the carcinogenic potency of the compounds studied (*15,16*). In other instances, no such correlation could be found (*17*). The assumption was made that, in these cases, secondary factors such as tumor promotion and/or progression ultimately determine whether a tumor will develop or not. In any case, knowledge about the induction and persistence or disappearance of specific DNA adducts will serve as a basis for the study of the relation between DNA damage and biological endpoints such as mutation induction and cancer. For obvious reasons, much of this knowledge will have to be derived from animal experiments. At present, methods are being developed which will permit analysis of DNA damage in human samples. Currently, such biomonitoring data may be used only to indicate that human exposure has taken place. Interpretation of these results in terms of a possible health risk for the exposed group or individual will require further experimentation and comparison with animal data (*18*).

In animal studies or in experiments with cultured cells, analysis of DNA adduct formation can be carried out by the use of radiolabeled genotoxicants (*19*). The amount of adducts can be determined directly on the basis of radioactivity present in isolated total DNA. Distinct types of adducts can be measured after chromatographic separation of enzymatically degraded DNA or through analysis of depurination products (*20–22*). Furthermore, adduct formation can sometimes be established by the measurement of radiolabeled excretion products in urine samples (*23*). In recent years, several methods have been developed to measure the presence of nonradioactive adducts in DNA, e.g., by means of postlabeling and chromatographic analysis of mononucleotide adducts obtained after enzymatic digestion (*24*), or immunochemically, with specific antibodies against the adducts (*25*). With these techniques, monitoring of human exposure to genotoxic agents will become possible.

This paper deals with some recent experiments in which polyclonal and monoclonal antibodies are used for immunochemical detection of DNA adducts in animal tissues and in human blood cells. Also, the development of a system to detect and quantitate adducts at the single-cell level will be illustrated.

Use of Polyclonal and Monoclonal Antibodies against DNA Adducts

Various sensitive immunoassays are now in use to detect DNA damage at extremely low levels (*26–29*). The sensitivity of immunochemical techniques depends largely on the affinity of the antibodies for their respective antigens or haptens. Also, a high antigen specificity is required for the selective detection of one type of adduct among other adducts or unmodified DNA components. Polyclonal and monoclonal antibodies of high specificity and affinity toward DNA adducts have been obtained (*30*). In an earlier publication (*31*), we discussed the heterogeneity of conventional polyclonal antisera with respect to specificity and affinity. The hybridoma technique (*32*) permits the isolation of monoclonal antibodies which are structurally and functionally homogeneous. This property is essential in experiments that require a low background antibody binding, such as the detection of a small amount of adducts among a vast excess of unmodified DNA components (see below). Recently, a method was developed in our laboratory to select and isolate hybrid cells that produce monoclonal antibodies of a desired specificity, by means of rosette formation (*33*) (Fig. 1). This method has the potential to select antibodies according to their affinity. An application of this selection technique is shown in a later section of this paper.

Different approaches towards immunochemical detection of DNA damage can be discerned. The first approach involves isolation of DNA from the exposed cultured cells or tissue and direct immunochemical detection of the adducts within the purified DNA or in an enzymatic digest thereof (*26*). Secondly, after digestion or depurination of the DNA, the products can be separated by means of chromatographic techniques, prior to immunochemical analysis (*34,35*). Under optimal conditions these techniques allow complete separation of the DNA adducts from other material, which results in a large decrease or even virtual elimination of undesired background antibody binding to the latter during the subsequent immunoassay carried out on the eluted fractions. A third approach aims at detection of DNA damage at the level of the single cell. In this case, exposed cells are fixed, and the DNA is denatured and sometimes deproteinized to enhance the accessibility of the adducts. The antibody must be able to bind adducts within the fixed DNA structure. Binding to nonmodified stretches of DNA and to cytoplasmic material should be minimal. This detection method can be applied to cells that are fixed while attached to glass slides or free in suspension (*36,37*). Finally, reports have appeared on visualization of adducts in individual DNA molecules by means of immuno-electron microscopy (*38*).

In the methods mentioned above, the antigen is exposed to the antibody in different structural environments (within the DNA, as free DNA adduct, etc). In principle, therefore, each of these approaches could require a different type of antibody. It may well be, that a polyclonal antiserum is sufficiently heterogeneous to be suitable for each type of immunodetection. However, monoclonal antibodies have a unique but restricted set of properties and the optimal antibody must be selected for the particular problem studied. In this connection it is worth mentioning that high affinity should not always be regarded as the only selection criterion to obtain the most suitable antibody preparation. It is also useful to design a hybridoma screening system that mimics the experimental conditions during the actual application of the antibody.

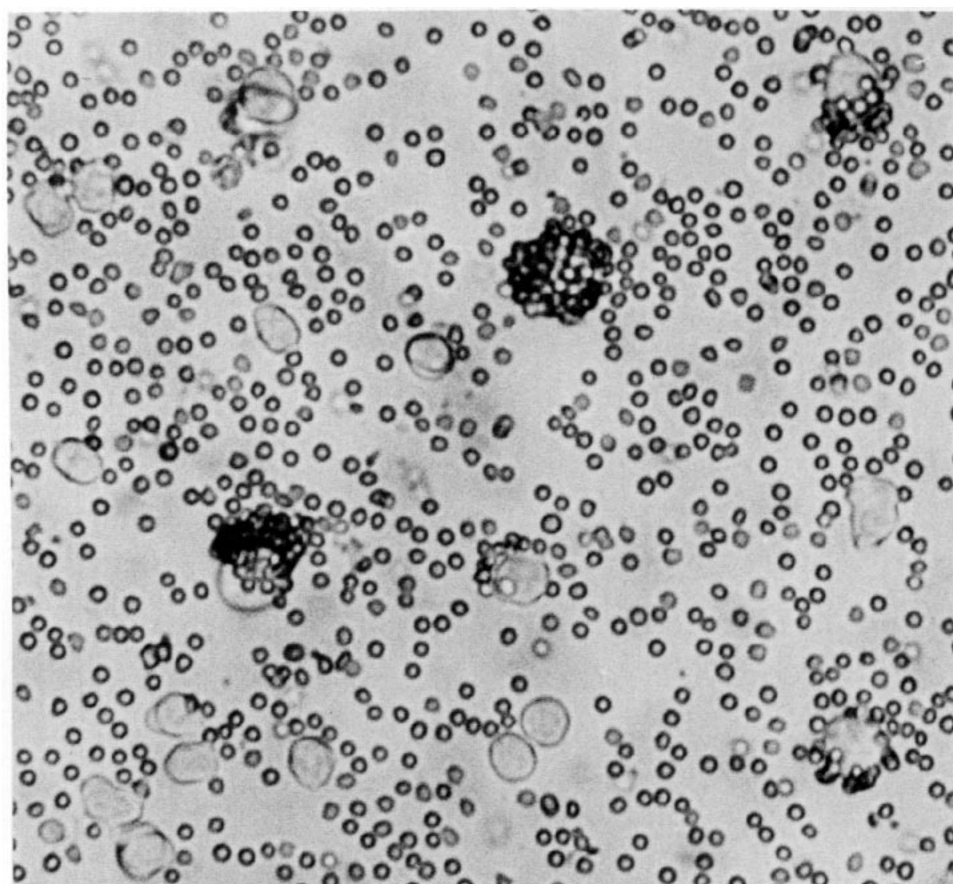

FIGURE 1. Selection of specific antibody-producing hybrid cells by means of rosette formation. Hybrid cells formed after fusion of myeloma cells with spleen cells from an immunized mouse were incubated in the presence of sheep erythrocytes, coated with hapten. Depending on the incubation conditions, the red blood cells formed rosettes, either by adhering to the antigen receptors on the surface of the hybrid cells or by forming a dense layer of red blood cells, due to the agglutinating action of the excreted antibodies. In both instances, single rosettes can be easily discerned, removed from the suspension with a micropipette, and grown monoclonally. In this way pure antibody-producing clones can be obtained from the fusion mixture in a single step. The picture shows rosette-forming hybrid cells, producing antibodies against the AAF-guanosine adduct (*31*). Experimental details of this selection technique have been published (*33*).

Some of these considerations will be illustrated below.

Organ-Specific Induction of AAF DNA Adducts in 2-AAF-Treated Rats

From animal carcinogenicity studies it is known that carcinogenic compounds may differ widely in their pattern of cancer induction sites within the animal or in animals of different species (*39*). Organ specificity of genotoxic chemicals may be due to differences in the capacity of the chemicals to reach the various organs or to differences between the organs in their capacity to bioactivate the chemicals or to repair the DNA lesions. By studying organ specificity of a certain DNA lesion one may obtain insight into the relevance of this lesion with respect to, e.g., tumor induction in various organs. Such a study can be useful in attempts to extrapolate animal data to man, for which no justifiable method is as yet available (*40*).

We have studied the induction of lesions in DNA from various organs of rats, exposed (PO) to various dosages of 2-acetylaminofluorene (2-AAF), which is a specific liver carcinogen in rats. Polyclonal antibodies (from rabbit) directed against AAF-modified guanosine (*31*) were used to measure the amount of AAF adducts in DNA from liver, spleen, and white blood cells. Only in liver DNA could extensive dose- and time-dependent induction of these adducts be detected (Table 1). No detectable amount of AAF damage was present in DNA from spleen or white blood cells.

To investigate whether induction of AAF damage in various organs is affected by the route of administration, a pilot experiment was carried out, in which the agent was administered by means of IP injection. Preliminary results show that also in this case, no adducts

Table 1. AAF adducts in liver DNA isolated from 2-AAF-treated rats.[a]

Dose, μmole/kg	AAF adducts/10^8 nucleotides			
	After 2 hr	After 6 hr	After 24 hr	After 48 hr
0	0	0	0	0
0.45	ND	ND	ND	ND
4.5	2	20	50	15
45	10	40	300	800
900	40	160	420	930

[a] Rats were treated (PO) with 2-AAF, dissolved in DMSO (5 mL/kg). After 2, 6, 24, and 48 hr, animals were killed; DNA was isolated from the livers and purified by means of CsCl equilibrium density gradient centrifugation. The number of AAF-adducts (given as number of adducts/10^8 nucleotides) was determined immunochemically in a competitive ELISA (*58*). ND = not detectable; the detection limit in this assay is 1 adduct/10^8 nucleotides.

could be detected in spleen DNA. In the liver, a lower level of AAF–DNA adducts was observed than after oral administration of the chemical.

Biomonitoring of Genotoxic Damage in Humans: Analysis of DNA from Cancer Patients Treated with Genotoxic Cytostatics

Extrapolation from animal carcinogenicity studies to the human situation is very difficult, not only because of the variation observed in cancer induction in various animal organs and species, but also because large differences have been demonstrated among human individuals in their metabolizing enzymes and their response to foreign chemicals. Some of these differences are partially of genetic origin (*41*). It is therefore important to gather as much information as possible from those cases in which humans are exposed to genotoxic agents. One possibility is to study the effects of antineoplastic drugs used in cancer chemotherapy. Several of these agents are genotoxic, and it is assumed that their antitumor activity is based on their interaction with DNA. Patients who receive this type of drug often respond quite differently to the treatment. Therefore, knowledge about the DNA damage induced by genotoxic antitumor agents may help to design a dosing regimen for individual patients, so as to minimize the clinical side effects and improve the therapy. Such studies could also lead to the synthesis of novel analogs of the drug, displaying equivalent or higher antitumor activity and causing less severe side effects.

We have studied the various adducts induced in DNA—*in vitro* and in cultured prokaryotic and eukaryotic cells—by the antitumor agent (*cis*)-diamminedichloroplatinum II (*cis*-DDP or cisplatin) (*42*). The adducts were isolated by means of anion exchange chromatography of an enzymic digest of (*cis*)-DDP-treated DNA, and several adducts could be identified by means of high-frequency proton NMR spectroscopy (*43,44*). A widely used method to analyze platinum-containing material is atomic absorption spectroscopy (AAS). The detection limit of this technique is often not sufficiently low to permit detection of DNA-bound platinum in, e.g., mammalian cells treated with *cis*-DDP in dosages that allow > 10% survival of the cells (*45*). Therefore, methods involving immunochemical detection of *cis*-DDP–DNA adducts are being developed (*46*). We have raised antibodies against the synthetic haptens *cis*-DDP–dGuo–dGMP and *cis*-DDP–Guo–GMP, coupled to bovine serum albumin. With these antibodies, several major *cis*-DDP–DNA adducts can be quantitatively determined in very small amounts. An example of the separation and immunochemical analysis of *cis*-DDP–DNA adducts in a digest of (*in vitro*)-treated salmon sperm DNA is given in Figure 2. By means of AAS, the presence of four different *cis*-DDP-containing (oligo)nucleotides could be established (Fig. 2*A*). Two of these adducts could be measured with high sensitivity in an immunoassay, with the two antisera mentioned above (Fig. 2*B*). Recently, we have started experiments to analyze the pattern of *cis*-DDP-induced adducts in DNA from human blood cells, i.e., from patients treated with cisplatin. With AAS, no platinum-containing material could be detected. However, with the method illustrated in Figure 2, the presence of the adducts Pt-GG and G-Pt-G (Fig. 2*A*) could be unambiguously demonstrated. With antibodies raised against *cis*-DDP–DNA (*46*)—which probably recognize the Pt–GG adduct in intact DNA—*cis*-DDP adducts could also be detected in DNA from nucleated blood cells of patients receiving *cis*-DDP (*47*).

Detection of DNA Damage in Single Cells by Means of Immunofluorescence Microscopy

Methods for biomonitoring human exposure must be very sensitive because the amount of DNA damage may be extremely small and because the amount of sample available is limited. On the other hand, the methods should be rapid and convenient to be suitable for large-scale screening purposes. Recently, several methods have been developed to measure DNA damage in individual cells with specific antibodies against DNA adducts, in combination with fluorescence detection. These methods involve the analysis of fixed cells, either on glass slides or free in solution. Although the latter method—flow cytometry—in general has been limited to the analysis of cell surface antigens, recent developments indicate that intracellular detection of specific DNA damage will become possible (*48*). The other method, immunofluorescence microscopy, involves immobilization of cells or tissue slices on glass slides, denaturation of DNA and detection of adducts with specific anti-adduct antibodies and second antibodies carrying

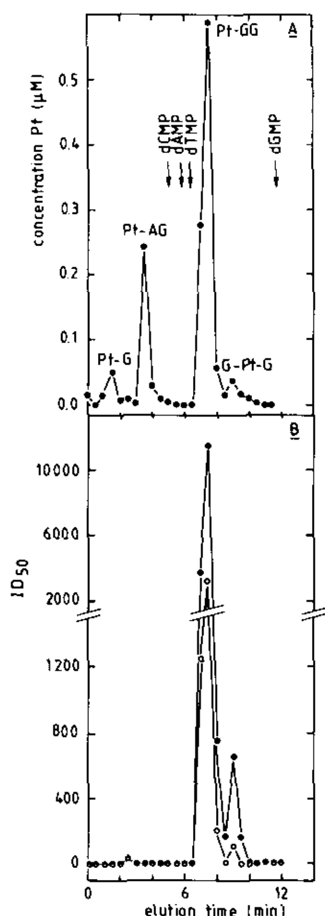

FIGURE 2. Chromatographic separation and immunochemical detection of *cis*-DDP-adducts. A digest of *cis*-DDP-treated salmon sperm DNA (15 μg) was chromatographed on an anion exchange column (MonoQ, Pharmacia, pH 8.8). The amount of platinum was measured in each fraction by means of AAS (panel *A*). The position of four identified platinum-containing adducts is indicated (*44*). Arrows indicate the elution position of the four common mononucleotides. In a competitive ELISA, each fraction was analyzed for the presence of platinum-containing adducts (panel *B*). The response is expressed as the dilution of the fractions, necessary to obtain 50% inhibition of antibody binding (ID_{50}). The two antisera used in this experiment (●,○) do not recognize the Pt-G and the Pt-AG adduct. Experimental details will be published elsewhere.

a fluorescent marker or an enzyme that converts a substrate into a precipitating, colored product. With this method, the morphology of the cells may be preserved, allowing the localization of specific DNA damage in distinct cell types (*49*). We have focused attention on the development of this method for the detection, with highly specific polyclonal antibodies, of AAF-adducts in cells treated with *N*-acetoxy-AAF (N-AcO-AAF), a reactive metabolite of 2-AAF. An advanced computer system designed for quantitation of the fluorescence signals is connected with the fluorescence microscope. Experimental details and the initial results obtained with this system will soon be published. An example of a dose–response curve constructed with computer-generated data is shown in Figure 3. We have also used monoclonal antibodies in this approach to detect AAF adducts at the single-cell level. Several monoclonals, isolated some time ago (*31*), failed to yield specific fluorescence signals on N-AcO-AAF-treated cells. This was ascribed to the fact that these antibodies had a 30-fold lower affinity constant than did the anti-AAF antiserum (from rabbit) which yielded a positive response. Recently, a large collection of different monoclonal antibodies was obtained by isolation, with the rosetting technique (Fig. 1), of hybrid cells producing antibodies directed against AAF-guanosine adduct. More than 50 cell culture supernatants were tested in the immunofluorescence microscopy system to visualize the adduct in mammalian cells treated with a rather high dose of N-AcO-AAF. Several of these yielded specific fluorescence signals. The affinity constants of the antibodies, as determined in a competitive radioimmunoassay (*50*), varied between 2×10^7 and 5×10^9 L/mole. Of the monoclonals that were negative in the single-cell assay, some also had a high affinity constant. It should be noted that all of the antibodies tested in this system recognize the free AAF–guanosine adduct or/and AAF-modified DNA, immobilized on an ELISA plate.

These findings illustrate the importance of a proper screening system to select antibodies with binding properties that are optimal for the problem under study.

Discussion

Biomonitoring of human exposure to environmental genotoxic agents requires advanced methods to detect very small quantities of DNA adducts in limited amounts of sample. Because environmental exposure involves nonradioactive compounds, the recently developed immunochemical techniques that are able to detect nonlabeled adducts with a high degree of sensitivity, will be very useful for biomonitoring purposes.

Chemically well-defined adducts have been used in immunogens to obtain highly specific antibodies and various types of sensitive immunoassays are now available to detect and quantitate the DNA adducts. The level of sensitivity that is achieved in these experiments warrants the expectation that monitoring of human exposure to genotoxic agents will become feasible (*51*). Recent developments described in this paper will enable us to analyze DNA damage, even quantitatively, in a limited number of blood cells. This is important in view of the need of large-scale screening methods for biomonitoring purposes. It should be noted that this immunochemical approach is feasible only in those instances where exposure results in the formation of known or identifiable adducts. Antibodies directed against groups of related DNA adducts would be useful for monitoring exposure to certain classes of chemicals (e.g., alkylating agents, polynuclear aromatics), without the need of knowing the identity or relative abundance of each separate compound. Monitoring of exposure to suspected but otherwise wholly unknown chemicals could be first

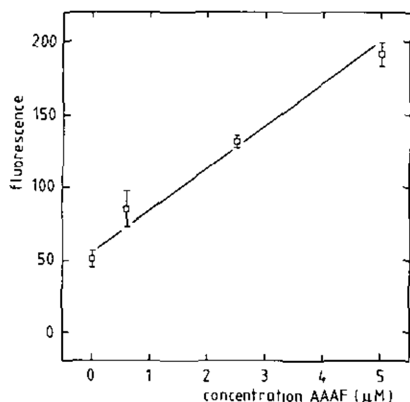

FIGURE 3. Quantitative immunofluorescence microscopy of DNA-adducts formed in cultured human fibroblasts treated with *N*-acetoxy-2-acetylaminofluorene. The cells were grown on cover-slips, treated with the agent for 1 hr at 37°C, washed and fixed with methanol/acetone. The preparations were treated with RNAse, proteinase K and alkali (0.07 N NaOH for 2 min). The slides were then incubated with anti-GuO-AAF antiserum (*31*) and FITC-labeled second antibody. The fluorescence was measured at the single-cell level (each point is the average of 35–40 cells).

approached with the postlabeling method described by Randerath (*24*). This method is nonspecific for the chemical and may be able to pick up the formation of any kind of adduct, under appropriate conditions. After isolation and identification of seemingly important, predominant or persistent adducts, as revealed by this method, the immunochemical approach could be chosen for further analysis.

When biomonitoring data are used only to establish effective exposure (i.e., resulting in damage in nuclear DNA), any DNA adduct that persists for a reasonable length of time may serve as a dose monitor. For monitoring of human exposure, the most readily available source of DNA are the white blood cells. Demonstration of the presence of DNA adducts in the blood is qualitative proof that exposure to a genotoxic agent has taken place. However, the presence of adducts in blood cells gives no clue about exposure of target organs such as liver and lung, which are—for obvious reasons—not readily available for experimentation. Furthermore, the absence of detectable DNA adducts in the white blood cells does not disprove exposure. Adducts may be rapidly removed from blood cell DNA or the reactive agent may not be able to reach the blood cells in sufficient quantity. Additional data—e.g., from *in vitro* exposure of human blood cells or from animal experiments—are required to study this problem in more detail. Our experiments with rats treated with 2-AAF indicate that no detectable amount of AAF-adducts was present in DNA isolated from white blood cells, even after administration of rather large doses of the carcinogen. In this case, the amount of adducts in blood cell DNA does not reflect at all the extent of exposure at the target organ, i.e., the liver.

It is clear that a negative result in a biomonitoring assay on blood cell DNA is not necessarily indicative of a nonexposure or "safe" situation. At the same time, additional research is necessary to provide a basis for interpretation of positive biomonitoring data in terms of a possible health risk for the exposed groups or individuals. Experiments with animals, in which additional data can be obtained with destructive methods, will remain indispensable, if risk assessment is the primary goal of biomonitoring. Such experiments may reveal which of the various adducts is responsible for the induction of the genetic effects observed (i.e., the key lesion). Although total adduct formation in DNA in some cases could not be correlated with tumor formation (*17*), it may well be that a specific type of lesion among the various adducts that are induced is directly related to the development of neoplasia or the occurrence of heritable disorder (*52*). Total adduct formation, e.g., measured in various organs after treatment of an animal with radiolabeled genotoxicants, does not necessarily reflect the relative abundance of these key lesions, because various types of damage may follow different, organ-specific kinetics of formation and repair. The relative amount of such lesions may thus be time-dependent. Among the various adducts induced in DNA of cisplatin-treated mammalian cells, the level of interstrand cross-links—assumed to be harmful lesions—does not run parallel with the total platination when measured at various intervals after the treatment (*45*). Comparison of *in vivo* animal data, e.g., on the fate of the key lesion and the ensuing genetic effect, with *in vitro* results obtained with animal and human cells in culture may allow an estimation of the expected effects in humans *in vivo* (*53*). The immunochemical methods enable us to approach these problems with great precision, because adducts of various types can be studied separately when the appropriate antibodies are available.

Recent data concerning mutational events at particular sites on certain chromosomes indicate that not only the mere presence of a specific lesion, but also its location in a certain region within the genome may be of importance for the development of genetic effects (*54*). The presence of a chemical lesion induced by the carcinogen benzo(a)pyrene diol epoxide was shown to convert a proto-oncogene into a transforming oncogene (*55*). Recombinant DNA techniques could be combined with sensitive immunochemical detection methods to elucidate the pattern of damage induction in various regions of the DNA, e.g., within specific fragments generated by digestion with restriction enzymes (*56*). This would contribute to our knowledge of the mechanism of carcinogenesis.

Exposure of humans to genotoxic agents offers the opportunity to study directly the human *in vivo* situation. Treatment with genotoxic cytostatics (e.g., platinum-containing antitumor compounds) generally occurs under well defined conditions with respect to the patient's diet, smoking habits and other medication. Several of these drugs have been identified as potential carcinogens, while some have been shown to induce second tumors in patients treated with chemotherapy (*57*). Material obtained from these people may therefore be

used to study the possible relation between certain DNA adducts and human cancer induction. In general, studies dealing with human exposure may be useful to develop methods for extrapolation of animal data to man and for calibration purposes.

The immunochemical approach thus appears to be a powerful tool in solving the problems encountered in monitoring human exposure to genotoxic agents. The current developments in this field may ultimately contribute to a sound scientific basis for the assessment of health risks for the exposed individual.

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