Measurement of Macromolecular Adducts as Indicators of Carcinogen Exposure

R. COLIN GARNER

1 INTRODUCTION

Cancer incidence varies from country to country, area to area, even town to town, as has been established from epidemiological studies. That finding suggests that much human cancer is influenced by environmental factors. It is not the purpose of this monograph to review the literature relating to human epidemiology. Rather, it is the objective to examine current methodology that could be used to monitor exposure, possibly at a cellular level, of individuals to substances responsible for initiating cancer.

Cancer induction is complex and certainly not fully understood. Agents known to cause human cancer have been separated into six groups:

(1) Irradiation, e.g., X-rays or ultraviolet light;
(2) Inorganic metals, e.g., arsenic and nickel;
(3) Hormones, e.g., diethylstilboestrol and anabolic steroids;
(4) Viruses, e.g., Epstein-Barr virus and herpes virus;
(5) Organic chemicals, e.g., benzidine and vinyl chloride; and
(6) Physical carcinogens, e.g., asbestos.

Group 5 is probably the largest group contributing to total cancer incidence. Groups 4 and 5 are merging through the elucidation of mechanism(s) of action of oncogenes. Of the six classes of carcinogen, those in categories 1, 2, 4, and 5, it could be argued, exert their effects directly through interaction with DNA; those in the other two groups may have a direct DNA damaging effect, but this has yet to be elucidated. This chapter will be directed towards human exposure to organic chemicals and how such exposure may be monitored using a variety of novel techniques.

2 MECHANISM OF CARCINOGEN ACTION

To devise a strategy for monitoring human carcinogen exposure, it is necessary to have some understanding of carcinogen metabolism. Figure 1 sets out a commonly accepted scheme for the process. At each point, a

Methods for Assessing Exposure of Human and Non-Human Biota. Edited by R.G. Tardiff and B. Goldstein

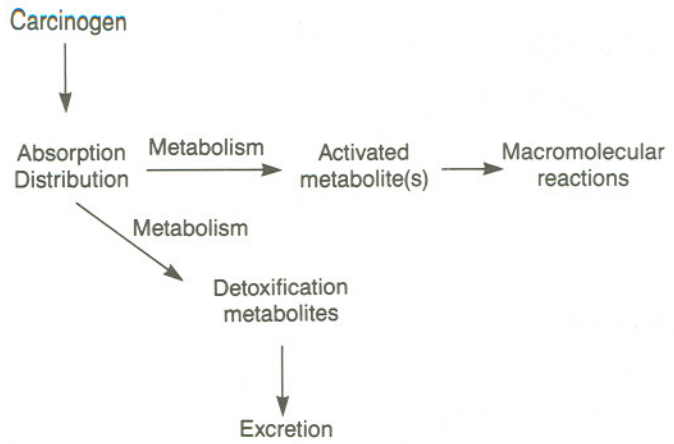

Figure 1. Simplified scheme for carcinogen metabolism

variety of factors such as nutritional status, species, sex, age, and disease status can affect the rate at which each reaction takes place.

In Figure 2, a more specific example of the complex processes whereby a carcinogen is metabolised in the body are described. Aflatoxin B_1 is the example of choice, since many of the metabolic routes for this mycotoxin have been elucidated. Inter-individual differences in carcinogen susceptibility could be related to the balance between activation to AFB_1 8,9-oxide and detoxification to metabolites such as AFP_1.

The activation and detoxification of carcinogens occurs in a variety of organs but is probably most extensive in the liver. The enzymes concerned are primarily mono-oxygenases which catalyze phase 1 oxidative metabolism and phase 2 conjugation metabolism. Many organic chemical carcinogens appear to be metabolised through similar processes to those I have described for AFB_1. This process of activation to an electrophilic metabolite has been demonstrated for such diverse structural groups as the nitrosamines, the polycyclic aromatic hydrocarbons (PAH), aromatic amines, and halogenated hydrocarbons. Many macromolecular adducts of these carcinogens have been structurally identified, as have some of the mechanisms by which cells recover from such damage.

The interaction of a carcinogen with DNA (a fundamental property of many carcinogens which are activated to electrophiles) is thought to be an important, if not essential, step in the cancer process. Current hypotheses suggest that carcinogen reactions lead to mutational events within the cells, leading finally to a loss of growth control. Molecular biological methods using restriction mapping, base-sequencing, and gene probing all support this view of cancer initiation. Reactions with DNA, therefore, should

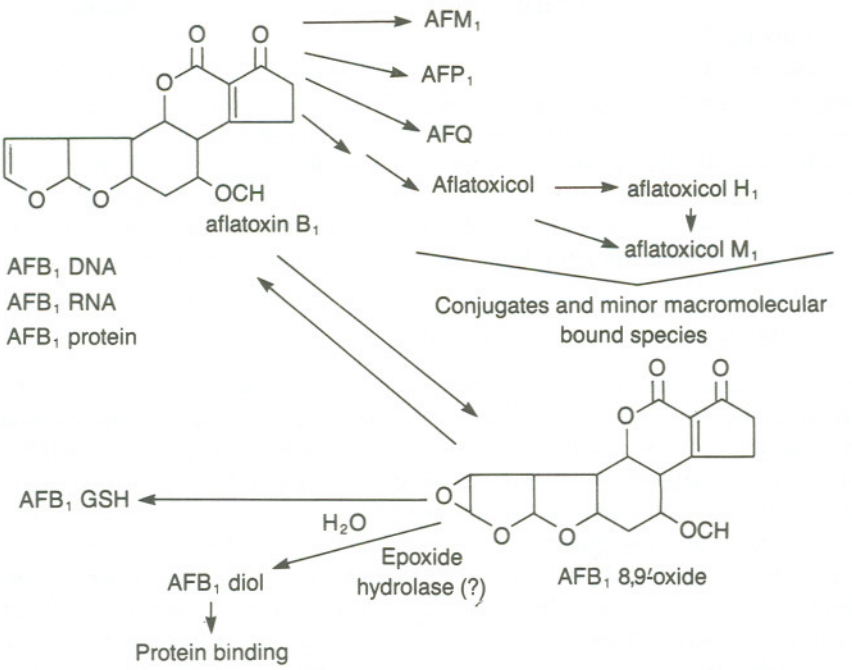

Figure 2. Scheme of aflatoxin B_1 metabolism

provide a means of studying critical events for the cancer process at a molecular level in both animals and humans.

3 STUDIES IN HUMANS

Considerable scientific controversy exists relating results of animal carcinogenicity experiments to humans. Examples include the hepatocarcinogenicity of halogenated hydrocarbons and a variety of liver enzyme-inducing agents. Do results in animals relate to exposure in humans? Until recently, it was not possible to contemplate laboratory-based methods which might answer this question. Many of the human risk assessment procedures involve extrapolation from animal data, despite the fact that differences in pharmacokinetics and pharmacodynamics are known to exist. How sure are we that an animal's response to a high dose of chemical extrapolates to that at low doses? Can we be sure that rates and routes of metabolism, and macromolecular binding are linearly related to dose? Such questions cannot be answered in humans using the same techniques used routinely in animal experiments. One could not conceive of injecting large doses of radiolabelled

compound in people and removing tissues or organs to measure macromolecular binding levels.

This review is, therefore, concerned with non-invasive, rapid, inexpensive, sensitive, specific, and reliable methods for performing studies in man. Several conferences and reviews have been devoted to this topic, and the reader should refer to these for an overview of the subject (Garner, 1985). All the methods are designed to move experimental cancer research from an animal-based to a human-based subject. Weinstein has coined the term "molecular cancer epidemiology" to describe this research area.

3.1 MACROMOLECULAR BINDING

Many organic chemical carcinogens have been found, after administration to animals, to bind to cellular macromolecules. These macromolecular-bound species are usually formed at nucleophilic centres as a result of electrophile activation of the carcinogen, as outlined above. Numerous studies have been performed demonstrating the binding of carcinogens to DNA and the subsequent repair of such damage. Since reaction of an electrophile can occur with many nucleophilic centres, not only can one demonstrate carcinogens binding to DNA but also binding to RNA and protein. These latter two have not been as extensively studied as the former, since DNA reactions are thought to be of major importance in initiating tumors. Nevertheless, there is undoubtedly a relationship between protein, RNA, and DNA binding. Levels of binding to one macromolecule by a carcinogen might be used to develop human monitoring programs.

Carcinogen binding to macromolecules often occurs to the greatest extent in organs which subsequently develop tumors. However, there is no 100 percent relationship between DNA binding and subsequent tumor production. A good example is the case of ethylnitrosourea which, although it binds overall to a greater extent to liver DNA than brain DNA, induces tumors of the brain and not the liver. In this case it appears that the site of reaction on a particular base, the O^6-position of guanine, is an important step in cancer causation and is not among the inconsequential DNA reactions. In other words, studies to monitor exposure to carcinogen should recognise that not all DNA reactions need be deleterious.

Nevertheless, for certain compounds, there is a good correspondence between the level of DNA binding and subsequent tumor induction. In the case of ionizing radiation, this relationship has led to recommendations of international safety standards, in which the levels of radiation thought to be biologically tolerable are presented. For chemical carcinogens, organisations are striving to develop similar safety standards, based on scientifically sound criteria. So far, this has been difficult to achieve, since there are a plethora of factors that can affect levels of DNA binding as a result of carcinogen exposure. For radiation, the DNA damage induced is direct,

i.e., so many rads will induce a certain amount of DNA damage. With chemicals, although one can determine exposure levels, little information exists on what exposure leads to an unacceptable degree of DNA damage.

3.2 MEASUREMENT OF MACROMOLECULAR BINDING

In animals, the determination of macromolecular binding is by radioactivity measurement after administration of a radiolabelled chemical. Provided one can obtain the test chemical in a pure radioactive and stable form, then assaying of binding levels is reasonably routine. For human tissues, one can use only indirect methods with a radiolabelled chemical. Studies have been conducted on human cultured cells and organ explants to demonstrate that human tissue behaves similarly to animal tissue. If DNA adducts of similar structure are obtained, then *a priori* humans have the same activating enzymes as the susceptible animal species and are, therefore, susceptible. This can lead to studies which demonstrate AFB_1 can bind to human lung DNA, even though there is no convincing evidence that this mycotoxin is a human lung carcinogen.

Recently, a number of procedures have been developed which could prove useful in the measurement of macromolecular carcinogen-bound species in man. These are described below.

3.2.1 Antibody Methods

Antibodies can be exquisitely sensitive and specific for their antigen. This fact has been used for many years in clinical chemistry to measure low concentrations of analyzed substances in human body fluids. Antibodies have been raised in sheep, rabbits, and goats routinely for clinical immunoassay. The production of antibodies against DNA adducts was first reported in the mid-60's, but little attention was paid to these early reports. It has only been with the introduction of monoclonal antibody technology that interest in antibody techniques in general has been rekindled. The early reports concerned the production of antibodies to DNA photoproducts induced by ultraviolet light. DNA is a notoriously poor antigen, and immunisation procedures had to be adapted to augment the antibody response. The original publications concerned the generation of polyclonal antibodies in rabbits. Little attention has been paid to the sensitivity and specificity of these antibodies. The pioneering work of Erlanger and his colleagues and of Poirier indicated clearly the vast potential of antibodies for DNA adduct screening.

A typical scenario for human monitoring would be to obtain antibodies against an adduct of interest, develop an appropriate immunoassay procedure, and then analyse for adducts in suitable human tissues or body fluids. While procedures can be described in a few words, the reality is that it may take

months, if not years, to carry out the process. Antibodies can be generated against DNA adducts when bound to DNA, to the isolated DNA adduct, or to the carcinogen moiety. Each of these procedures has been used to prepare useful antibodies.

Antibodies Against DNA Adducts As already mentioned, immunisation with DNA does not generally lead to high antibody titres. The situation can be improved by co-immunizing not only with Freund's adjuvant, but also with methylated bovine serum albumin or methylated keyhole limpet haemocyanin. The co-injection of this protein appears to generally activate the B-cells in the spleen, rendering them more responsive to the weak DNA immunogen. Methylation of this protein enables the DNA to attach electrostatically and so stabilise the complex.

Polyclonal antibodies have been generated utilizing these methods against benzo(*a*)pyrene diol epoxide-reacted DNA, acetoxyacetylaminofluorene reacted DNA, *cis*-diamminedichloroplatinum(II) reacted DNA (*cis*-DDP-DNA), DNA- containing thymine dimers generated by ultraviolet light and aminofluorene-reacted DNA. Some of these antibodies have been used to analyze human DNA. In a series of analyses of human lung DNA recovered from either surgical specimens or autopsy material, five of the samples had demonstrable benzo(*a*)pyrene DNA adducts. All patients had been heavy cigarette smokers. Analysis was by ELISA, which is becoming the method of choice for immunoassay. The chief problem with this particular study was that the results were obtained at the limit of sensitivity of the assay.

In another study using a polyclonal antibody to *cis*-DDP-DNA, no false-positive results were obtained from 63 individuals out of the 130 blood samples taken; i.e., only lymphocyte DNA from *cis*-platin-treated individuals had measurable adducts. An increasing accumulation of *cis*-DDP-DNA adduct levels was observed in individuals given repeated doses of the drug.

Monoclonal antibodies have been prepared from benzo(*a*)pyrene diol epoxide-reacted DNA, aflatoxin B_1-reacted DNA and imidazole-opened aflatoxin B_1. No reports have been published on the use of these antibodies to screen human tissue samples.

Antibodies Against DNA Adducts Conjugated to Proteins Since DNA is a poor immunogen, a number of workers have prepared conjugates of the isolated DNA-adduct with a protein such as bovine serum albumin. These conjugates have been used to prepare both poly- and monoclonal antibodies.

Polyclonal antibodies have been raised against O^6-methyl deoxy-guanosine, guanosine (8-yl) acetylaminofluorene, guanosine (8-yl) aminofluorene and O^6-ethyldeoxyguanosine. Many antibodies have been used to compare adduct levels in animal tissues as measured by radioactivity determinations and results by immunoassay. On the whole, a good correspondence has been obtained between the two procedures using adducted DNA obtained either from treated cell cultures or from animals dosed with the appropriate carcinogen. Monoclonal antibodies have been obtained using either immu-

nised mice or rats, and subsequent cell fusions against O^6-methyl, O^6-ethyl, and O^6-butyldeoxyguanosine, O^4-methylthymine, and O^2-methylthymine. Oesophogeal DNA from patients in high oesophogeal cancer incidence areas in China have been found to have detectable levels of O^6-methylguanine as measured using immunoassay techniques. Control populations had no detectable O^6-methylguanine levels.

Antibodies Against Carcinogens An indirect method to measure carcinogen adducts with DNA utilises antibodies generated against the carcinogen moiety. While this procedure enables adducts of differing structure to be detected, its non-specificity is a disadvantage when using body samples. Unless the DNA adducts are extensively purified first, then cross-reaction of the antibody will occur with both RNA and protein contaminants. Non-specificity of reaction might be advantageous in fishing expeditions, in which one wants to find out if a particular organ has any carcinogen-adducts. Antibodies against carcinogens can be either polyclonal or monoclonal, and are produced as a result of immunizing with a carcinogen + protein conjugate.

The author has studied extensively both animal and human samples to determine if they contain aflatoxin moieties subsequent to aflatoxin exposure. For these studies, we have utilised a polyclonal preparation prepared in rabbits, as a result of repeated immunisation with an aflatoxin B_1-bovine serum albumin conjugate. Using an ELISA method developed in our laboratories, we have examined human urine obtained from patients in The Gambia. Large variations in antibody inhibitory material were found, indicating that some of the samples did indeed appear to contain aflatoxin or its metabolites. While it is possible to obtain qualitative measurements using these methods, it has proved impossible to obtain quantitative information on unextracted urine. The reason for this is our finding that different aflatoxin metabolites or DNA adducts have different affinities for the antibody. Antibody binding to metabolites reflects not only their concentration but also different metabolite affinities. Individual differences reflect not only concentration differences but also metabolite affinities. The only way that this particular problem might be resolved would be to purify. individual metabolites by chromatography and assay fractions. This procedure would negate the advantages of immunoassay in terms of speed and simplicity.

Nevertheless, we have used the anti-aflatoxin B_1 polyclonal antibody to make comparisons between macromolecular binding in the rat as assayed by radioactivity and by ELISA. The results indicated that it should be possible to measure plasma protein binding in humans and to extrapolate to estimate levels of DNA binding. Since the antibody preparation was raised against an aflatoxin B_1 + protein conjugate, it has proved sensitive to aflatoxin B_1 moieties when bound to protein.

An extension of this approach is that reported by Sun and his colleagues.

They have prepared a monoclonal antibody against aflatoxin M_1 and used this to make an immunoaffinity column. When urine from people thought to have been exposed to aflatoxin was passed through the column and the bound material subsequently eluted, aflatoxin M_1 was detected. There was an approximately 1 percent conversion rate of aflatoxin B_1 to aflatoxin M_1 when measurements were made of aflatoxin B_1 in ingested beer and of aflatoxin M_1 excreted in urine. A similar immunoaffinity column procedure has been reported using an IgM monoclonal antibody of aflatoxin B_1; however, this has not been used for human studies.

3.2.2 Antibody Methods—The Future

For cancer to develop in humans, it usually requires continuous exposure to the carcinogen. Cigarette smoking provides a good example, since smoking a single cigarette is not likely to produce lung cancer. However, smoking twenty cigarettes daily for many years increases the risk of contracting cancer of the lung many times over that of a non-smoker.

Our ability to measure exposure over a long time period is limited by our present methodology. It would be desirable to find some biological disturbance that could reflect the cumulative effect of carcinogens. Our present methods suffer from their inability to provide anything more than single time point estimations of exposure. This is likely to be of little use in determining the reason for individual differences in susceptibility. An approach to get round this problem might be to collect all urine voided by an individual over the period of a month. This urine would be passed through an immunoaffinity column, and any of the antigen under study would be trapped. If the immunoaffinity column was prepared from an antibody against a carcinogen + DNA adduct, then it would be possible to measure the amount of this adduct excreted over the period of a month. The adduct amount should reflect the level of DNA damage over the month as well as the rate of repair of this damage. Such measurement could provide an estimation of the total body burden to a carcinogen.

Other estimations of carcinogen body burden could be derived from carcinogen reactions with haemoglobin or other plasma proteins or lymphocyte DNA. Such estimations, which could be by physico-chemical and/or antibody procedures, could provide the scientist with the necessary information to estimate risk. The measurement of these adducts will depend not only on whether they are formed, but also on their stability and longevity. To date little attention has been paid to the use of antibody methods to measure carcinogen-protein adducts, but these might prove a more useful method than techniques such as mass spectrometry.

3.3 ^{32}P POST LABELLING ANALYSIS OF DNA

Many carcinogens, when they react with DNA, will lead to adduct + base conjugates, which have different chromatographic properties when compared with their parent base. This principle has provided a novel procedure which has been primarily used for adduct analysis in animals. A method enzymatically digests extracted purified organ DNA to the monophosphates, using DNAse 1 and snake venom phosphodiesterase. The total DNA digest is then treated with polynucleotide kinase and ^{32}P[ATP]. The resulting bisphosphate is labelled with ^{32}P at the 5′ end. The labelled digest is then chromatographed on PEI-cellulose thin-layers using two-dimensional chromatography. Any carcinogen-base biphosphates will have different R_f values compared with the parent bases. Visualisation is by autoradiography. A variety of carcinogens has been examined using this technique, including polycyclic aromatic hydrocarbons, benzidine, 2-acetylaminofluorene, and safrole and related compounds. Disadvantages of the procedure are that an authentic carcinogen-base monophosphate is required as a chromatographic reference and that many carcinogen-base bisphosphates do not have a sufficiently different R_f value compared with the unreacted bases. This applies particularly to adducts that are substituted with small residues such as methyl and ethyl.

Digestion of lymphocyte DNA from a smoker might give rise to numerous spots on a TLC, which do not correspond with the normal bases. However, unless authentic standards are available, it is impossible to identify these spots. The limit of sensitivity of this method is approximately 1 in 10^6 bases, which is at the lower end of the sensitivity scale required to measure nucleic acid adducts in real-life situations. On the other hand, sensitivity is dependent solely on the specific radioactivity of the ^{32}P used.

3.4 MACROMOLECULAR BINDING MEASURED BY PHYSICO-CHEMICAL METHODS

There have been several published papers on the use of analytical methods to measure macromolecular adducts. Most attention has been directed toward studies on alkylated or arylated haemoglobin using mass spectrometry as the detection method. In this procedure, haemoglobin is obtained from animals or persons exposed to carcinogens, or presumptive carcinogens, and hydrolysed to amino acids. Crude separation of amino acids is carried out on ion-exchange columns, and the resultant separated products analyzed by mass spectrometry. Many carcinogens have been shown to react with haemoglobin in animal studies, suggesting that this approach may have common application. Since there are nucleophilic sites in haemoglobin, these are susceptible to attack by electrophilic carcinogens. As a result, substituted aminoacids are obtained which can be quantitated by mass spectrometry.

A useful illustration is that of ethylene oxide, a direct-acting mutagen.

Ethylene oxide reacts with histidine in haemoglobin to yield *N*-3-(2-hydroxyethyl) histidine. Levels of this substance have been correlated in animal studies with levels of alkylation of liver and testicular DNA. A correspondence has been found between liver binding and haemoglobin binding, suggesting that the latter can be used to monitor the former. While this approach is an elegant one, very few carcinogens to date have been studied. Reaction with haemoglobin will be dependent on the activated carcinogen actually reaching the target protein. This is going to be an unlikely event for highly reactive electrophiles. On the other hand, a carcinogen such as 4-aminobiphenyl, and possibly other aromatic amine carcinogens, actually attack the haemoglobin *in situ* because they can be activated by red blood cell peroxidases. 4-Aminobiphenyl adducts in haemoglobin can be detected using mass spectrometry in animals not thought to be knowingly exposed to 4-aminobiphenyl.

In addition to mass spectrometry of alkylated haemoglobins, one can use similar techniques to analyze for excreted macromolecular adducts in the urine. The sensitivity of the instrument means that very low levels of adduct might be detected after low-dose carcinogen exposure. 7-Methylguanine has been demonstrated in the urine of animals dosed with either dimethylnitrosamine or the precursors aminopyrene and sodium nitrate.

A combination of immunoaffinity chromatography for concentration and mass spectrometry for identification might prove a useful approach.

Fluorescence spectroscopy provides another method for examining adduct levels in human tissues. A number of carcinogens, such as benzo(*a*)pyrene and aflatoxin B_1, have characteristic and strong fluorescence spectra. With the introduction of powerful computers and Fourier transform analysis to separate signal from noise, adduct levels as low as 1 in 10^6 to 1 in 10^8 can be detected. It has been possible to demonstrate aflatoxin B_1 + guanine in the urine of persons thought to be exposed to aflatoxin B_1 in Kenya. DNA samples from alveolar macrophages obtained by lavage have been found to have detectable levels of benzo(*a*)pyrene + DNA adducts. Not only do certain carcinogens have characteristic fluorescence spectra but so also do some base adducts. O^6-Methylguanine, for example, has a stronger fluorescent spectrum than guanine. The etheno/adenine adduct formed on reaction of vinyl chloride epoxide with adenine also has a characteristic spectrum. Such physico-chemical properties can be exploited provided these are known in advance.

3.5 HUMAN TISSUES THAT CAN BE SAMPLED NON-INVASIVELY

Resourcefulness is needed to find tissues that can be used for monitoring purposes. Urine and faeces provide the easiest, and perhaps the least pleasant, of body fluids to obtain. Most subjects do not object to providing

specimens for analysis. Difficulties come in the storage of large numbers of samples or in the volume of samples for population-based studies. Blood samples are somewhat more difficult to obtain, require trained personnel, and are often removed for other purposes such as clinical chemistry. Other cells that may be used for analysis include those from the buccal lining of the mouth and exfoliated bladder cells, which have sloughed off into the urine. The latter can be obtained by centrifugation of the voided urine sample.

From all the cell types obtained as above, it is possible, using micro methods, to isolate sufficient quantities of DNA, RNA, and protein for analysis. Limits of sensitivity of detection procedures need, however, to be in the nano- to picogram range.

4 CONCLUSIONS

Various techniques have been described that enable one to estimate levels of carcinogen/macromolecular adducts in human tissues. Most of these procedures have sufficient sensitivity and specificity to estimate exposure in real, as opposed to artificial, situations. More effort and resources need to be applied to this research in the future if scientists are to relate results of animal studies to human cancer risk. In addition, the methods described should enable us to assess the importance of environmental chemicals in the aetiology of human cancer.

5 REFERENCE

Garner, R.C. (1985). Assessment of carcinogen exposure in man. *Carcinogenesis* **6**, 1071–1078.