Pergamon Press Ltd

GENETIC AND NONGENETIC EVENTS IN NEOPLASIA

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(Received 2 February 1981)

Summary—It has become increasingly evident that all chemical carcinogens do not act *via* the same mechanism of tumorigenicity. Based upon the extent of a chemical's interaction with DNA, a general classification scheme of various mutational and nonmutational theories of chemical carcinogenesis is presented. Compounds that directly interact with DNA are classified as genotoxic whereas those that do not interact directly with DNA are classified as epigenetic carcinogens. Under each general heading, several mutational and nonmutational mechanisms of carcinogenesis are believed to be possible. Data are also presented to support the existence of one such mechanism, an epigenetic-mutational theory of chemical carcinogenesis based upon recurrent cytotoxicity. In this case, increased regenerative DNA synthesis in response to tissue injury is believed to result in an enhancement of the normal spontaneous mutation rate, conceivably leading to a cellular transformation. The carcinogenic risk posed by such epigenetic carcinogens appears to differ greatly from that posed by genotoxic carcinogens. Thus, consideration of data concerning the possible mechanism of carcinogenicity of a chemical, along with pharmacokinetic data, will allow a better understanding of bioassay results and a more accurate assessment of carcinogenic risk.

Introduction

Frequently in the routine rodent carcinogenesis bioassay of a chemical high levels of a test compound are administered for prolonged periods of time and, at the termination of the study, the numbers of tumours formed are counted. If a statistically significant excess of a particular tumour is observed the compound is classified as an animal carcinogen and an extrapolation of risk is made to humans. Yet in most cases, without knowledge of the fate of the chemical in several species (i.e. pharmacokinetics) as well as of the resultant macromolecular events to help understanding of the bioassay results, there is little chance of a meaningful assessment of human carcinogenic risk. It is the integration of these three data bases (bioassay, pharmacokinetics and macromolecular events) to more realistically assess the potential carcinogenic risk of a given chemical that has been a continuing endeavour in our laboratory over the past several years. Recently, we have been involved in trying to understand the different mechanisms of tumour formation and their implications for risk assessment, and, more specifically, the role of cytotoxicity in the carcinogenic process. This latter area of research will be the focus of this paper.

Classification of proposed mechanisms of chemical carcinogenesis

Since the first experimental documentation of chemically induced carcinogenesis in animals by Yamagiwa & Ichikawa (1918), numerous theories have been proposed to explain the mechanisms of chemical carcinogenesis. Apart from the various differentiation, protovirus and viral infection theories of carcinogenesis (Temin, 1974), most theories regarding the mechanism(s) of chemical carcinogenesis may be generally classified as 'genetic' or 'epigenetic' (nongenetic) in nature. As discussed below, the distinction between these two general classes of mechanisms is of great significance in carcinogenic risk assessment. As used here, chemicals displaying a genetic mechanism of carcinogenesis are defined as those involved in direct interaction with cellular DNA. In most cases this interaction is considered to result in a mutational event; however there may also be cases of nonmutational genetic mechanisms of carcinogenesis (e.g. Holliday, 1979). Chemicals displaying an epigenetic mechanism of tumorigenesis are defined as those that do not directly interact with cellular DNA, and yet their action may indirectly result in a mutational event. Thus, the terms genetic and epigenetic in chemical carcinogenesis, as used here, refer to the likelihood of direct chemical–DNA interaction, and in either case may or may not involve a mutational event.

The most common interpretation of a genetic mechanism of tumorigenesis is embodied in the somatic mutation theory of carcinogenesis first proposed by Boveri (1929). Basically, this theory dictates that the direct interaction of a chemical with DNA (e.g. by alkylation, intercalation) can result in a somatic cell mutation (*via* point or deletion mutation, gene duplication or gross chromosomal disruptions) which may or may not ultimately lead to a transformed or neoplastic cell. As reviewed by Hanawalt, Friedberg & Fox (1978) mitigating factors in this progression are the DNA-repair enzymes which remove the chemically induced 'lesion' before (excision repair) or after (post-replication repair) DNA replication (i.e. before the lesion is fixed as a mutation). It also appears that errors in this repair, possibly by an inducible error-prone DNA-repair enzyme system analogous to SOS repair observed in bacteria, may be responsible for the mutation rather than the initial chemically-induced mispairing of bases (Kondo, 1976 & 1977; San & Stich, 1975; Sarasin & Benoit, 1980; Witkin, 1976).

Comings' (1973) 'general theory of carcinogenesis' provides a possible scenario of events leading to a state of anaplasia after a mutation occurs. In this case the significant events would be a double mutation of one or more regulatory genes (normally present in all cells), in turn derepressing corresponding structural genes capable of coding for cellular transforming factors. In the case of chemical carcinogenesis following an apparent two-step progression towards neoplasia ('two-step theory of carcinogenesis'), this derepression of genes following a mutational event (initiation) may be promoted by epigenetic stimuli, chemical or physical (Boutwell, 1974; Trosko & Chang, 1978).

Substantial evidence has been accumulated to link mutagenic events with the neoplastic transformation of cells. As reviewed by Trosko & Chang (1978), findings regarding the clonal nature of tumours, the mutagenicity of many carcinogens, the correlation of high cellular mutation frequency with increased cancer rates among humans suffering from deficiencies in DNA-repair enzymes, the correlation of *in vitro* DNA damage with cell transformation frequencies and neoplasia, the involvement of mutation in the initiation phase of some hydrocarbon-induced carcinogenesis, the effects of age on mutagenesis and the incidence of various hereditary tumours, are all supportive of a mutagenic origin of cancer. However, several findings cannot be reconciled with a mutagenic basis of carcinogenesis. These have included observations regarding the fact that all carcinogens are not mutagens (Jensen, 1974; Lippman, 1975; Segaloff, 1975), the discrepancy between gene mutations and cell transformation rates in short- *v.* long-lived animals (Huberman, Mager & Sachs, 1976; Peto, 1977), the induction of tumours by plastics- or metal-film implantation and hormone imbalance (Berenblum, 1978), the ability of teratoma cells to produce apparently normal cells upon transplantation into normal mosaic mouse blastocytes (Mintz & Illmensee, 1975; Papaioannou, McBurney, Gardner & Evans, 1975) and the ability of Lucké frog adenocarcinoma cells to reproduce normal cells upon nuclear transplantation into anucleate eggs indicating a totipotency of the tumour-cell genes (McKinnell, Deggins & Labat, 1969). Thus, there is also substantial evidence for the existence of one or more epigenetic nonmutational mechanism of carcinogenesis.

Several theories have attempted to reconcile these different mechanisms of carcinogenesis either by proposing an alternative to mutagenesis or by accommodating both genetic mutational and epigenetic nonmutational mechanisms. A recent example of the former situation is the nonmutagenic theory proposed by Holliday (1979). This theory proposes that gene regulation, as directed by specific DNA base methylation and recognition by several DNA methylases, may be altered during repair of DNA–chemical alkylation sites. The resultant loss of specific methylated recognition sites for DNA methylases results in loss of gene regulatory control and ultimately in a transformed cell. Since the direct interaction of the chemical with the DNA is required to deregulate genes in this nonmutagenic theory, it is still consistent with a genetic mechanism of tumorigenicity as defined above. An example of the latter 'coexistence' type of theory is the 'integrative theory' of carcinogenesis of Trosko & Chang (1978). These authors propose that neoplasms may arise from a mutagenic event alone (regulatory locus) if the genes affected are in a transcribable state, from a mutagenic event if there is a coupled nonmutational alteration of the mutated genes (promotion), or from the abnormal derepression of genes at critical developmental stages that prevent normal gene regulation.

Based upon the apparent realization that some chemicals may cause tumours *via* several different mechanisms of action (i.e. there is no single unifying mechanism), it has been possible to propose a classification scheme for chemical carcinogens even though mechanistic details remain to be elucidated. Weisburger & Williams (1980) have thus proposed that carcinogenic chemicals be categorized under the general division of genotoxic chemicals (direct-acting or primary carcinogens, procarcinogens or secondary carcinogens, inorganic carcinogens) and epigenetic carcinogens (solid-state carcinogens, hormones, immunosupressive agents, co-carcinogens, promoters). A diagrammatic representation of the various theories of carcinogenesis is presented in Fig. 1.

Concept of the cytotoxic mechanism of tumour formation

An epigenetic mechanism of tumorigenesis that is not frequently considered in terms of tumour production is that of recurrent cytotoxicity. The simplest example of this mechanism is the production of sarcomas following subcutaneous injection of inert solutions such as saline or glucose. It is envisaged that repeated subcutaneous injection of these nonreactive materials results in inflammation, necrosis and cellular division which may increase the error rate in normal DNA replication or DNA repair, resulting in critical-site mutation and ultimately in a transformed or neoplastic cell. In general terms, this theory represents a modification of Virchow's 'irritation theory' in which hyperplasia was believed to be the driving force behind carcinogenesis (Berenblum, 1944). It is likely that some chemicals may illustrate both genetic and epigenetic effects. In such an instance it will be important to determine which mechanism is predominantly responsible for the formation of tumours.

With the concept of testing maximum doses in animals in order to enhance the probability of detecting a carcinogenic response, one of the primary issues is the relevance of such testing in terms of the real risk for humans of a chemical shown to be carcinogenic under such test conditions. Frequently in such studies tumours are observed at dose levels that also cause recurrent cytotoxic responses, and a logical question would be whether an analogy to sarcoma production following subcutaneous injection may be made. In understanding this relationship, it is enlightening to explore the molecular basis for increasing mutation as a consequence of increased DNA synthesis and cell division.

DNA does not exist *in vivo* as a pristine molecule even in the absence of a measurable genotoxic challenge. So-called spontaneous mutations appear to occur at a rate dependent upon exposure to unavoidable exogenous (e.g. cosmic radiation) and endogen-

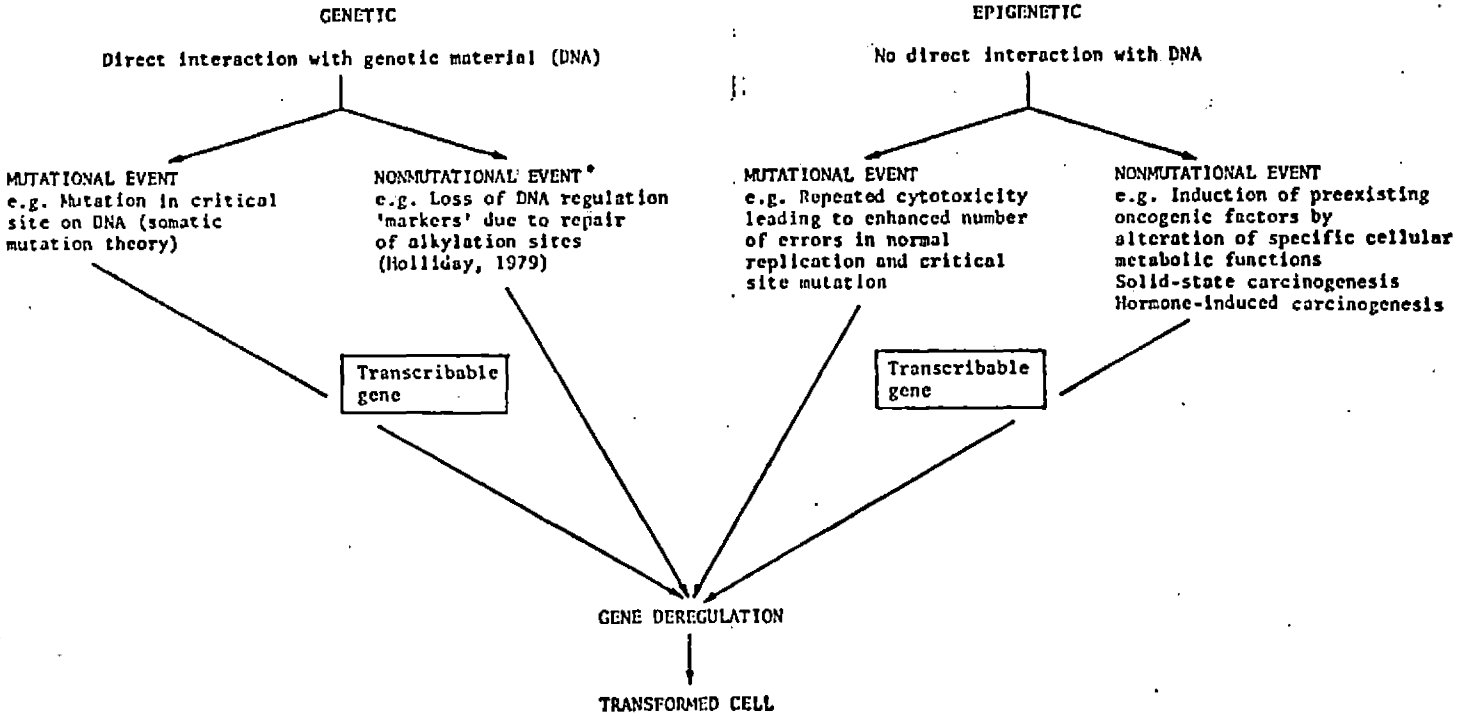

Fig. 1. General classification of some of the proposed mechanisms of carcinogenesis, excluding the differentiation, oncogenic, viral infection and protovirus theories. The role of DNA repair in mutagenesis is not included.
*Believed to be a readily reversible alteration (Holliday, 1979).

ous factors, DNA-repair competency (e.g. relative to age) and the basic thermo-stability of the DNA molecule itself. With regard to the latter point it has been estimated that thermal decomposition may account for between 2000 and 10,000 depurinations, several hundred depyrimidinations and several base deaminations of DNA molecules in a mammalian cell per generation (Lindahl & Karlstrom, 1973; Lindahl & Nyberg, 1972 & 1974). These thermal lesions can result in base transitions (e.g. deamination of 5-methylcytosine to form thymine) which conceivably may be 'fixed' (irreparable) by DNA replication or repair processes resulting in a functional mutation. Indeed, errors in the process of DNA replication itself may occur as a result of base mispairing, DNA polymerase base selection errors and mismatch repair errors (see review by Hartman, 1980). Based upon estimates of the mutation rates per human gene per sexual generation, it has been suggested that 10% of all human gametes contain a new mutation of their own plus several inherited mutated genes as well (Drake, 1978). Thus the mere fact that DNA synthesis and cellular division is enhanced following cytotoxic responses will result in a decreased time for repair of 'naturally' occurring DNA lesions before replication, an increased number of replication errors, and an enhanced somatic mutation rate.

Additionally, since errors in DNA may occur throughout the cell cycle or during DNA synthesis, the DNA-repair process can be thought of as a continuous process. However, as noted, some forms of DNA repair are error-free while others may be error-prone. Therefore the fidelity of DNA repair can affect the somatic mutation rate during cell division. A recent report by Shank & Barrows (1980) on studies of the noncarbonatious chemical hydrazine has shown that cytotoxic doses of hydrazine cause abnormal methylation of nucleic acid bases in the liver. Furthermore, evidence was presented which suggests that at hepatotoxic doses other chemicals (e.g. carbon tetrachloride) produce a similar alteration in the DNA as a result of cytotoxicity. Whether this effect is due to an alteration in the DNA polymerase or in the fidelity of DNA repair following DNA replication is not known, but it is an example of how a cytotoxic agent at high dose levels can cause abnormal nucleic acid bases to occur in cellular DNA.

DNA synthesis and cell division can also enhance the susceptibility to mutation by exogenous and endogenous agents. For example, Berman, Tong & Williams (1978) have reported an increased mutation frequency in actively dividing adult rat-liver epithelial cells exposed to the mutagenic chemical methyl methanesulphonate compared with that in quiescent (nondividing) cells. In this study the number of azaguanine resistant colonies per 10^6 colony-forming units was used as a measure of mutation frequency. The mutation frequency in the actively dividing cultures was increased five-fold when compared with the quiescent culture, indicating that dividing cells were more susceptible to mutation.

Finally, DNA synthesis and cell division can also enhance mutation frequency by altering the amount of DNA repair prior to DNA replication. An example of this is given by the work of Maher, Dorney, Mendrala, Konze-Thomas & McCormick (1979) using normal human fibroblasts and fibroblasts lacking normal DNA excision repair. Cell survival and mutation rate in cells exposed to ultraviolet (UV) radiation was observed to be related to DNA-repair competency. Survival of normal cells was higher and mutation rate lower per UV dose than for deficient cells. Normal cells were also able to survive a usually lethal and mutagenic UV dose by being held in confluence (nondividing) for a period of time before assessment of survival and mutation rate, presumably because this allowed a greater time for DNA repair to occur before replication.

In addition to a molecular basis for enhancing mutagenic events as a result of increased DNA synthesis, there is an experimental basis for the role of DNA synthesis in carcinogenesis. Tumours often develop in chronically inflamed or scarred tissue; colon cancer is frequently observed in patients with chronic colitis; skin cancer occurs in burn scars; liver tumours are associated with chronic cirrhosis of the liver (Berenblum, 1944; Chan, 1975; Laroye, 1974). Repeated tissue damage with a physical agent (dry ice) and resultant regeneration has induced tumours in mice (Berenblum, 1929). Physical trauma such as partial hepatectomy has also been shown to enhance the tumorigenic effect of thioacetamide, *N*-nitrosodimethylamine (NDMA) and *N*-nitrosodiethylamine (Craddock, 1978; Date, Gotoskar & Bhide, 1976). Dimethylbenzanthracene-induced skin tumours promoted by the classic phorbol esters have been observed to be inhibited by anti-inflammatory steroids which inhibit inflammation, DNA synthesis and cell proliferation (Slaga, Fisher, Viaje, Berry, Bracken, LeClerc & Miller, 1978; Weeks, Slaga, Hennings, Gleason & Bracken, 1979). Finally, as noted above, tumours can be induced at the site of subcutaneous injection of nonreactive chemicals, such as glucose, saline or distilled water (Grasso & Golberg, 1966).

Having explored the molecular as well as experimental basis for the effects of cytotoxic responses on the carcinogenic process, the obvious question arises about the impact of cell division and increased DNA synthesis on genotoxic chemical carcinogens. For genotoxic carcinogens it is likely that cytotoxicity will cause an increased incidence and/or a decreased latency in the production of tumours. A good example of this is the induction of liver tumours by NDMA (Terracini, Magee & Barnes, 1967). At low doses of NDMA that preclude cytotoxic effects in the liver, tumours can still be induced. At higher levels where both cytotoxic as well as genotoxic effects act in concert, liver tumours are observed at an increased frequency as well as at a decreased latency. For chemical carcinogens displaying a cytotoxic mechanism with little to no genotoxic activity it is likely that a prolonged, recurrent cytotoxic response throughout a large portion of the animal's life will be required for the induction of tumours.

The implications of these carcinogenic mechanisms are as follows. (1) For genotoxic carcinogens, the defence mechanisms such as DNA repair, detoxification and excretion of the reactive species will be critical for modifying the carcinogenic response. Risk estimates need to consider the dose-related kinetics of the lesions produced and the extent and persistence of those genetic lesions. (2) For epigenetic carcinogens

causing tumours *via* a cytotoxic mechanism of tumorigenicity, tumours will be induced at doses that produce recurrent cytotoxicity and no carcinogenic risk would be predicted at doses that preclude any tissue injury.

It is expected that chemical carcinogens will represent a spectrum of activity ranging from genotoxic to epigenetic. Those chemicals having strictly genetic activity are often those that either are, or can be activated to, strongly electrophilic species. Chemicals with intermediate activity may have both a genetic as well as an epigenetic component to their tumorigenic response and some chemicals that show little to no genetic activity may produce tumours strictly through the mechanism of recurrent cytotoxicity or other epigenetic mechanisms. This is not to say that chemical reactivity is always directly relatable to carcinogenic potency but only that such compounds show a propensity to react with macromolecules. Obviously, the qualitative character or reaction site specificity of these reactions will have a profound impact upon the outcome of the DNA–chemical interaction. Indeed, a majority of interactions would not be expected to result in any biologically significant event because they involve noncritical-site alkylation or repair. It is also important not to rule out endogenous factors such as genetic predisposition working in concert with epigenetic mechanisms since all species and strains are susceptible to the development of particular tumours that are not related to exogenous chemical exposure. This concept is of paramount importance in assessing the relevance of extrapolating test results in sensitive animal species to humans.

Experimental approaches: some examples

The experimental approach taken in our laboratory has been to develop some objective criteria to differentiate between the genetic effect and the epigenetic, cytotoxic mechanism of tumour production. The two parameters that were selected as representative of a genetic effect were (1) DNA damage, measured by alkylation of DNA *in vivo* and (2) DNA repair, induced presumably by damage to the DNA molecule. While there are inherent limitations to using total DNA alkylation as an index of genetic interaction, due to the overall lack of information on specific critical sites of reaction on all of the nucleic acid bases, it was one of the only means of assessing the potential for *in vivo* genetic interaction. NDMA was selected as a genetic-acting carcinogen and the extent of DNA alkylation and of DNA repair following treatment with the test chemicals were compared with those after NDMA treatment. NDMA was also selected because it represented an electrophilic methylating agent and the chemicals we were testing were low-molecular-weight molecules. If high-molecular-weight heteroaromatic molecules are being tested, benzo[*a*]pyrene or the naphthylamines may be appropriate. For evidence of epigenetic, cytotoxic effects, DNA synthesis in the target tissue was measured and classic histopathology was also carried out. The chemicals that were tested were perchloroethylene, chloroform and 1,4-dioxane.

The results of the carcinogen bioassay on perchloroethylene conducted by the National Cancer Institute and of studies conducted in our laboratory are summarized as follows. Mice exposed by gavage to approximately 500 or 1000 mg/kg/day throughout their lifetime showed an increase in liver tumours (hepatocellular carcinomas), but rats exposed to equally high doses did not show any tumorigenic response (National Cancer Institute, 1977). In inhalation studies, exposure to 300 or 600 ppm perchloroethylene daily for 1 yr (subsequent observation for 18 months) did not prove to be tumorigenic in rats (Rampy, Quast, Leong & Gehring, 1978). It is important to emphasize that the $B6C3F_1$ mice in which liver tumours were enhanced by perchloroethylene treatment have an average control incidence of hepatocellular carcinoma of 10–15% (some nearly 50%). Therefore, this species and strain has a marked genetic predisposition for liver neoplasms.

Studies designed to characterize differences in the pharmacokinetics of perchloroethylene in rats and mice showed the following (Table 1; Schumann, Quast & Watanabe, 1980; Watanabe, Reitz, Schumann, McKenna, Quast & Gehring, 1980). When exposed to a 10 ppm perchloroethylene atmosphere for 6 hr mice metabolized and activated nine times more perchloroethylene than did rats. This resulted in a seven-fold greater macromolecular binding in the liver of mice than rats. Importantly, binding to hepatic DNA in mice at a tumorigenic dose level of 500 mg/kg was not observed. The detection limit for the perchloroethylene–DNA binding studies in mice was 10 alkylations per 10^6 nucleotides.

When parameters of cytotoxicity were assessed (Table 1) the liver weights were increased in treated mice but not in rats. DNA synthesis caused by tissue injury was increased by 82% in the mice but was not significantly increased in rats, and finally histopathological alterations in the liver were evident in mice but not in rats.

Another possible indication of direct genetic interaction is that of gross chromosomal damage and of *in vitro* mutagenesis, if a possible nonmutational gene deregulation mechanism of carcinogenesis (e.g. Holliday's theory) is ruled out. When mutagenicity data for perchloroethylene were examined (Table 2), it was noted that negative results in Salmonella have been reported from three laboratories and there has been one report of a positive finding in the Ames-type strain of *Salmonella typhimurium*. Further, in a cytogenetics study no chromosomal abnormalities attributable to perchloroethylene exposure were observed. The overall conclusion is that perchloroethylene has little to no genetic activity.

In summary of the perchloroethylene data, DNA alkylation was not detectable at a detection limit of ten alkylations per 10^6 nucleotides; DNA-repair assays were not conducted because they are generally less sensitive than the alkylation studies. Mutagenicity data is predominantly negative in bacterial systems as well as negative in an animal cytogenetics study. Cytotoxic responses indicated by increased DNA synthesis, two-fold over that of control, and histopathological examination were evident in the mouse at tumorigenic dose levels. In contrast, a classic genotoxic carcinogenic agent (e.g. NDMA, ethylnitrosourea) would alkylate DNA at a level of hundreds to thousands per 10^6 nucleotides; DNA repair would

Table 1. *Perchloroethylene metabolism, genotoxicity and cytotoxicity data (Schumann, Quast & Watanabe, 1980; Watanabe, Reitz, Schumann, McKenna & Gehring, 1980)*

Perchloroethylene treatment	Parameter	Response in Rats	Response in Mice
Inhalation exposure to 10 ppm for 6 hr	Total metabolized (μmol-equiv/kg body weight)	10·5	89·5
	Total macromolecular binding (μmol-equiv/g hepatic protein)	0·02	0·147
Single oral dose of 500 mg/kg body weight	Hepatic DNA binding	—	ND
Twelve oral doses of 500 mg/kg body weight/day	Liver weight:body weight (% increase)	5	25
	Hepatic DNA synthesis (% increase)*		
	Hepatic histopathological changes†	−	+

ND = Not detected

*Present as a percentage increase in DNA synthesis in treated compared with control animals as measured by [6-^{3}H]thymidine uptake.

†A treatment-related response was observed (+), or no treatment-related response was observed (−).

illustrate a dose-response relationship; *in vitro* mutagenicity would be clearly evident. In contrast to these genotoxic effects, there would be little histopathological alteration or increased DNA synthesis in the target organs at low, though tumorigenic, dosages, thus providing evidence of a lack of a significant role of an epigenetic, cytotoxic mechanism. Our overall conclusion is that the liver tumours observed in mice following lifetime administration of high doses of perchloroethylene were a result of the recurrent hepatotoxic effect of perchloroethylene which caused an enhancement of the normal background liver tumour incidence in a strain of mice with genetic predisposition towards the development of such tumours. Most importantly, hepatotoxic effects in animals and humans would be observed only at dose levels that are well above levels encountered in the occupational environment. Thus the relevance of the data that has been obtained for mice which suggests a carcinogenic risk to humans exposed at occupational or environmental levels of perchloroethylene is highly questionable.

The next example is that of chloroform (Reitz, Quast, Stott, Watanabe & Gehring, 1980). The carcinogenicity data for male mice given chloroform are summarized in Table 3. This represents a compilation of more than one study, but these data clearly show that following administration of chloroform at high doses male mice are susceptible to the development of liver and kidney tumours. It is noteworthy that at 17 mg/kg/day no significant incidence of tumours at any site was observed. At high doses chloroform also induces tumours in the rat, but for the sake of space we shall limit discussion here to mouse data.

At an oral dose level of 240 mg chloroform/kg body weight, mouse liver and kidney DNA was alkylated at a rate of three and one alkylations per 10^6 nucleotides respectively (Table 4). These values represent the upper bounds of chloroform alkylation since no actual DNA adducts have been identified. In contrast,

Table 2. In vitro *mutagenicity data and cytogenetics data on perchloroethylene*

Bioassay system	Results*	Reference
Escherichia coli	−	Greim, Bonse, Radwan, Reichert & Henschler (1975)
Salmonella strains	−	National Institute for Occupational Safety and Health (1977).
	+	Černá & Kypěnová (1977)
	−	Bartsch, Malaveille, Barbin & Planche (1979)
	−	National Toxicology Program (1980)
Cytogenetics	−	Černá & Kypěnová (1977)

*Positive (+) and negative (−) responses are indicated.

Table 3. *Data from tumorigenicity assays of chloroform given orally to male mice*

Chloroform dose	Strain	Percentage of excess tumours (site)	Reference
277 mg/kg/day, 5 days/wk for 78 wk	$B6C3F_1$	92 (liver)	National Cancer Institute (1976)
60 mg/kg/day, 6 days/wk for 80 wk	C57BL	ND	Roe, Palmer, Worden & Van Abbé (1979)
60 mg/kg/day, 6 days/wk for 80 wk	ICI	23 (kidney)	Roe *et al.* (1979)
17 mg/kg/day, 6 days/wk for 80 wk	ICI	ND	Roe *et al.* (1979)

ND = Not detected

a dose of 10 mg NDMA/kg results in approximately 540 alkylations per 10^6 nucleotides in the liver. Similarly, a tumorigenic dose of 240 mg chloroform/kg was not observed to induce hydroxyurea-resistant DNA repair (Table 4) while NDMA showed a dose-related increase in DNA repair following doses of 3, 10 and 20 mg/kg body weight.

Cytotoxicity, as evidenced by increased DNA synthesis in both the liver and kidney of chloroform-treated mice, showed an increasing dose-response relationship at 60 and 240 mg chloroform/kg body weight, but it was normal at a nontumorigenic dose of 15 mg/kg (Table 4). Concomitant with the increase in DNA synthesis, histopathological alterations were observed at the higher dose levels. On the other hand, NDMA, at 3 mg/kg body weight, showed no propensity towards increasing DNA synthesis or histopathological alterations as evidence of cytotoxicity.

In summary of the chloroform data, DNA alkylation in the liver following a tumorigenic dose level of chloroform was a maximum of three alkylations per 10^6 nucleotides. DNA repair was not detectable. A review of the literature has also shown that chloroform is not mutagenic in a mammalian cell and several bacterial mutagenesis assay systems (Kirkland, Smith & Van Abbé, 1981; Reitz *et al.*, 1980). Evidence of chloroform's cytotoxicity has come from studies using mice dosed with a tumorigenic level of 240 mg/kg body weight. DNA synthesis was observed to be increased 14-fold in the liver and 25-fold in the kidneys of these animals, and this was associated with histopathological observations of toxicity. It appears that the genetic potential of chloroform is nil to very little, while the component of recurrent cytotoxicity is very significant. Thus, as with perchloroethylene, it appears that chloroform causes tumours in mice *via* a cytotoxic mechanism rather than by a purely genetic mechanism.

The final example is that of 1,4-dioxane. When chronically administered to rats at a concentration of 0·75 to 1·8% in their drinking-water, 1,4-dioxane has been observed to cause an excess incidence of nasal and hepatocellular carcinomas (Argus, Arcos & Hoch-Ligeti, 1965; Hoch-Ligeti, Argus & Arcos, 1970; Kociba, McCollister, Park, Torkelson & Gehring, 1974). Ingestion of lower doses (0·1%) in drinking-water (Kociba *et al.* 1974) and inhalation of 1,4-dioxane (Torkelson, Leong, Kociba, Richter & Gehring,

Table 4. *Data on chloroform and N-nitrosodimethylamine (NDMA) genotoxicity and cytotoxicity in male CD-1 mice (Reitz, Quast, Stott, Watanabe & Gehring, 1980)*

Treatment	Hepatic DNA: Binding (alkylations/10^6 nucleotides)	Hepatic DNA: Repair*	DNA synthesis† in: Liver	DNA synthesis† in: Kidney	Histopathological changes‡ in: Liver	Histopathological changes‡ in: Kidney
Chloroform						
240 mg/kg	3	ND	×14	×25	+	+
60 mg/kg	—	—	×2·2	×8·2	−	+
15 mg/kg	—	—	ND	ND	−	−
NDMA:						
20 mg/kg	—	7·37	—	—	—	—
10 mg/kg	540	3·04	—	—	—	—
3 mg/kg	—	1·60	ND	—	−	—

ND = Not detected

*Ratio of treated control values. Values > 1·0 indicate an increase in DNA repair.
†Presented as multiples of control values as measured by [6-^{3}H]thymidine incorporation.
‡A treatment-related response was observed (+) or no treatment-related effect was observed (−).
Chloroform was administered as a single oral dose; NDMA was given as a single ip injection.

1974) have failed to produce excess tumours. At a tumorigenic dose level (1015 mg/kg/day or 1% in water), 1,4-dioxane was also observed to cause pronounced hepatic degenerative and regenerative changes (Kociba *et al.* 1974).

No genotoxicity has been observed in Sprague–Dawley rats dosed with a single oral carcinogenic dose level of 1,4-dioxane (1000 mg/kg); no increased DNA repair or hepatic DNA alkylation was detected. *In vitro* mutagenicity assays carried out following the methods of Ames, Durston, Yamasaki & Lee (1975) and using *S. typhimurium* strains TA98, 100, 1535, 1537 and 1538 also gave negative results. No DNA repair was detected in an *in vitro* primary rat hepatocyte RNA repair assay following the method of Williams (1977). Yet, as shown in Table 5, repeated administration of a carcinogenic dose level of 1,4-dioxane (1000 mg/kg body weight/day) was observed to be cytotoxic to rat hepatic tissue as evidenced by a 1·5-fold increase in hepatic DNA synthesis and abnormal histopathology relative to the controls. No cytotoxic changes were observed in rats dosed with a nontumorigenic level of 1,4-dioxane (10 mg/kg body weight/day).

The lack of genotoxicity and the observed cytotoxicity of 1,4-dioxane at tumorigenic dose levels indicates that as with perchloroethylene and chloroform in the mouse, 1,4-dioxane may cause tumours in the rat *via* an epigenetic-cytotoxic mechanism of action. However, the pronounced hepatocellular hypertrophic response observed in rats exposed to a high dose level of 1,4-dioxane (Table 5) and the observation that 1,4-dioxane induces hepatic drug-metabolizing enzyme systems in the rat (this laboratory) also suggest another epigenetic mechanism of tumorigenic action characteristic of metabolic inducing agents such as phenobarbital. It has been suggested that this mechanism of action may be a result of degranulation of rough endoplasmic reticulum resulting in alterations in protein synthesis and gene expression or by a hyperplasia-induced enhancement of the expression of pre-existing oncogenic factors (Tennekes, 1979; Wright, Akintowa & Wooder, 1979). Thus from the available data it appears that repeated exposure to high doses of 1,4-dioxane may cause tumours in rats *via* one or more epigenetic (mutational or nonmutational) mechanisms of action.

Conclusion

Obviously, much work remains to be done to fill in the gaps in our understanding of the mechanisms of chemical carcinogenesis. Future work to define critical sites on macromolecules (DNA, histones, nonhistone proteins) that may be important in the process of gene expression and cellular transformation will be of particular importance. However our understanding of the mechanisms of chemical carcinogenesis has reached a point whereby this knowledge may be used to generally classify carcinogens and in human risk assessment, even though the more definitive mechanistic details remain to be elucidated. The studies cited above, and others, have shown that all carcinogens do not act by the same mechanism. Some, which have genetic mechanisms of carcinogenicity, pose greater risks than others, which act by epigenetic mechanisms. Finally, it should be stressed that chemicals having an apparent cytotoxic mechanism of action are tumorigenic only at toxic dose levels, have reversible cytotoxicity, require multiple dosages, and appear to have realistic thresholds.

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Table 5. *Data on cytotoxicity of 1,4-dioxane in Sprague–Dawley rats*

Treatment	Dose (mg/kg/day)	Liver weight: body weight*	Hepatic DNA synthesis*	Hepatic histopathology†
Exposure *via* drinking-water for 11 wk	10	×1·00	×1·23	−
	1000	×1·12	×1·50	+

*Expressed as multiples of the control value.

†Treatment-related effects were observed (+) or no treatment-related effects were observed (−).

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