THE DEVELOPMENT OF TOXICOKINETIC AND TOXICODYNAMIC DATA FOR IMPROVING THE CHARACTERIZATION OF TRICHLOROETHYLENE'S EFFECTS AT LOW DOSES

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ABSTRACT

The ability of trichloroethylene (TCE) to induce liver tumors in B6C3F1 mice has underpinned the development of environmental standards for the past two decades. Alternatives to linear extrapolation require demonstration that a mode of action is involved that is unlikely to have a linear dose-response relationship. Our laboratory approached this problem along two distinct paths. The first path involved the investigation of the formation and further metabolism of the metabolites postulated responsible for the induction of liver cancer by TCE, dichloroacetate (DCA) and trichloroacetate (TCA). The second path was to determine if modes of action could be demonstrated that would not require linear extrapolation for purposes of risk assessment. Sufficient TCA is produced in the metabolism of TCE to account for a substantial fraction of the liver tumors. DCA is also produced at very low concentrations in blood, but it is much active at much lower concentrations in vivo than TCA. It appears that DCA could account for the remaining tumors.

TCA acts as a peroxisome proliferator. It has been argued that this class of carcinogens is not active in humans based on several grounds, the most persuasive being that its effects are mediated through the peroxisome proliferator activated receptor (PPAR) because the response can be blocked by disrupting the receptor with knockout mice. We found that that while DCA induces peroxisome proliferation, this does not appear to be its primary mode of action. The tumors produced by DCA are distinct from those produced by TCA. We have shown that DCA appeared to stimulate the growth of spontaneously initiated liver cells from mice on soft agar, suggesting that its weak mutagenic activity is not requirement for its tumorigenic effects. These results were confirmed *in vivo* by showing that the primary mode of action of DCA was to stimulate the rate of tumor growth utilizing magnetic resonance imaging. This was very consistent with our data showing that DCA had a selective ability to induce cell replication within preneoplastic lesions while depressing replication in normal cells. Modeling of these results suggest that tumor initiation by DCA is produced by suppression of apoptosis in a spontaneously mutated cells and fixation of these cells by stimulation of cell division.

These results strongly suggest that DCA or TCA does not involve linear mechanisms in the induction of liver cancer. Since the tumors can be entirely accounted for by these two metabolites, induction of liver tumors by TCE does not require low-dose linear extrapolation.
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INTRODUCTION

Trichloroethylene (TCE) is a halogenated solvent that is very commonly found in ground water near waste sites. It is the solvent that occurs most widely and at the highest concentrations at DOE sites (1).

Clean-up targets for TCE are driven by the maximum contaminant limit (MCL) for drinking water (2). The formal basis of this MCL is the practical quantitation limit (PQL) as defined by the EPA at the time of promulgation. However, this concentration closely approximated the projected one additional cancer per 1,000,000 population per lifetime at the upper 95% confidence limit calculated by the linearized multistage model. This calculated risk was based upon the production of liver tumors in B6C3F1 mice at doses of 1000 to 2000 mg/kg body weight for most of their lifetime (>78 weeks) in a series of studies conducted by the National Cancer Institute and National Toxicology Programs (3,4). Other organ sites have been affected in other species or when TCE was administered by a non-oral route. However, liver tumor induction essentially drives the risk assessment to values of dose that are almost one order of magnitude lower than other sites.

Three metabolites of TCE can also produce liver cancer in B6C3F1 mice, dichloroacetate (DCA), trichloroacetate (TCA) and chloral hydrate. Early authors had made the assumption that the liver tumors induced by TCE could be entirely be attributed to formation of TCA since it is the metabolite that is measured at the highest concentrations in blood and urine of both experimental animals and humans. However, several isolated pieces of data appear inconsistent with the observation that TCA was solely responsible. The most telling inconsistency was a closer correspondence in the mutation frequency and spectra in the H-ras protooncogene of tumors produced by DCA and TCE than between TCA and TCE (5,6).

Changes in mutation frequency and spectra in tumors from treated animals relative to tumors from control animals have been suggested as an indication that a chemical may be acting as a genotoxic agent (5). This hypothesis has come under some criticism because tumor promoters tend to decrease the mutation frequency in tumors from treated animals relative to control. However, we have pursued this question more in terms of identifying a common pattern of response to trichloroethylene and its metabolites to determine if the pattern seen with TCE was more closely identified with that produced by TCA or DCA. A complication of prior data is that TCE was administered to mice dissolved in corn oil, whereas animals were treated with DCA and TCA in their drinking water. Corn oil has been shown to modify carcinogenic responses to other solvents.

The proposed new cancer risk assessment guidelines of the U.S. Environmental Protection Agency open the door for considering less than linear extrapolation for modes of action that do not involve mutation. In some cases (e.g. chloroform) the establishment of alternate modes of action has led to proposals of non-zero maximum contaminant goals (MCLGs) for carcinogens (7).

The objectives of this research was to:
1. Clarify the relative contribution of the metabolites to liver cancer induction by TCE in mice.

2. Determine the mode of action for the responsible metabolites to see if non-linear extrapolation was appropriate for liver cancer.
3. Develop a database that is appropriate for modeling pharmacokinetic and pharmacodynamic factors that would be operative for TCE at low doses.

METHODS

The methods reported in this manuscript have been largely described in publications that have or will soon appear in the peer-reviewed literature. The methodology is both complex and varied and believed to of limited value to the audience. Consequently, only the methods are described in the most general of terms geared towards explaining the intent of measurements rather than describing methods in detail. The reader will be referred to the appropriate manuscripts for greater experimental detail.

Pharmacokinetic analyses of intravenous and oral administration of TCE, DCA, TCA and chloral hydrate and have been performed in mice, rats and to a lesser extent in humans. The most relevant to the data discussed in this manuscript is work conducted in mice (8,9,10) and humans (11).

Experimental studies in animals have generally involved the administration of DCA or TCA in the drinking water at concentrations of up to 2 g/L (12). Because of its limited solubility, TCE has been administered as an aqueous emulsion in Alkamuls®. Exposure duration varied according to the experiment described in the results from as little as on week to up to 80 weeks in some tumor studies. In the case of experiments where MRI imaging was done, the treatment of some mice was suspended to allow the effect of continued treatment on tumor growth rates to be evaluated (13). Preparation of slides, histological methods, including immunohistochemical staining are described in Stauber and Bull (12).

Sequencing of codon 61 of the H-ras gene was done by amplifying DNA from tumors using the polymerase chain reaction (PCR). After amplification the products were purified using Microcon 100 filter units and sequenced on an automated cycle sequencer. Sequencing was done in both directions (e.g. 5' to 3' and 3' to 5') for confirmation.

Growth of colonies of cells derived from primary mouse liver on soft agar is described in Stauber et al. (14). The important aspect of this assay is that normal cells do not grow on soft agar so what is picked up in these colonies are cells that were spontaneously initiated in animals. In this case, the treatments with DCA were included in the soft agar itself. The growth of these colonies with precisely the same phenotypes seen in tumors in mice treated with DCA and TCA *in vivo* is particularly important in differentiating effects of DCA from those of TCA.

Statistical analyses were performed according to the design of specific experiments. In general these could involve full analysis of variance to simple separation of means by a t-test. These descriptions are well laid out in the published manuscripts and the appropriate references have been identified.

RESULTS:

The critical pharmacokinetic issue to be addressed in our research was to identify the levels of dichloroacetate that were produced in the metabolism of TCE and to compare these data to the concentrations of DCA that lead to the development of cancer. DCA was important because it is a multispecies liver carcinogen, whereas the major metabolite TCA is only carcinogenic in mice (16,17,18), not in rats (19). Prior work had indicated that very large amounts of DCA were formed in the metabolism of TCE (20). However, subsequent research suggested that these high levels were produced artifactually from TCA during the preparation of samples for analysis (21).

Table 1 summarizes a large body of pharmacokinetic work performed to provide more realistic estimates of the levels of DCA that could be anticipated from doses of TCE that were used in bioassays as previously published in Barton et al. (10). These data are compared to measurements of DCA levels in the blood of mice that had been treated with subthreshold (0.05 g/L) and effective (0.5 g/L) doses of DCA in drinking water. DCA is very rapidly metabolized in the liver of non-pretreated mice which precludes measurement in blood at low doses above the limit of quantitation of 1.9 μM. The model was based upon a series of papers that described the pharmacokinetics of both DCA and TCA in mice and concentrations achieved in blood at dose of 0.5 g DCA/L and above (8,22). There are limitations that should be placed on the interpretation of these data. The comparison is based upon blood levels because that is the data that was available and this allows for a reasonable approximation to be made. In the case of a chemical that is metabolized as rapidly as DCA, however, it would be better to make the comparisons on concentrations in the liver. This is the obvious next step in developing a more comprehensive pharmacokinetic model, especially one that one would want to scale to a human model using appropriate kinetic data for the relevant human processes.

Table 1. Comparison of the area under the curve (AUCL) for concentrations of dichloroacetate (DCA) in the blood of mice treated with DCA directly in drinking water to the AUCL predicted to arise from doses of trichloroethylene used in the cancer bioassays of trichloroethylene (10).

Treatment	AUCL (mg-h/L)
0.05 g DCA/L of drinking water	0.041
0.5 g DCA/L of drinking water	0.72
1000 mg TCE/kg body weight	0.25
2000 mg TCE/kg body weight	0.31

However, the data are useful in that they place the amount of DCA predicted to be produced from bioassay level doses of trichloroethylene in the range that suggests DCA may be making

some contribution. It is important to note that these levels are not sufficient to account for all the hepatic tumors produced by trichloroethylene and that TCA is the probable cause for the remainder of the tumors that are induced. This observation was confirmed by the observation of Schroeder et al. (23) that H-ras codon 61 mutations were actually decreased in DCA-induced tumors in female mice. Thus, there is no support for the hypothesis that changes in the mutation spectra at this codon is an effect induced by DCA.

Since DCA could be present in carcinogenic amounts, it was not sufficient to understand the mode of action of TCA, but also of DCA. Previous work from our laboratory had demonstrated that the phenotypic characteristics of tumors induced by DCA and TCA are quite different (12). This apparent difference in mechanism is important because TCA is a peroxisome proliferator and such compounds have been considered as rodent-specific carcinogens because of the relative insensitivity of non-rodent species, including to humans, to the pleiotropic effects of this class of chemicals (24). Further research was needed to provide additional confirmation of differences in the tumors produced by DCA and TCA and to determine whether DCA's effects could be attributed to a non-genotoxic mode of action.

The mutation frequency and spectra in codon 61 of the H-ras protooncogene in tumors from historical control and chronic bioassay mice administered TCE dissolved in corn oil are provided in Figure 1. These historical data are provided for comparison with newer data obtained from experiments conducted in our laboratory with TCA, DCA and TCE administered in an aqueous vehicle. This last group seemed necessary in our evaluation because the published mutation frequency for tumors induced by TCE administered in corn oil appeared unusually high for compounds that act as tumor promoters (5). A further problem in the data that was available was that the published mutation frequency and spectra for TCA involved much too few tumors (6) to provide statistical confidence in the estimates that were available.

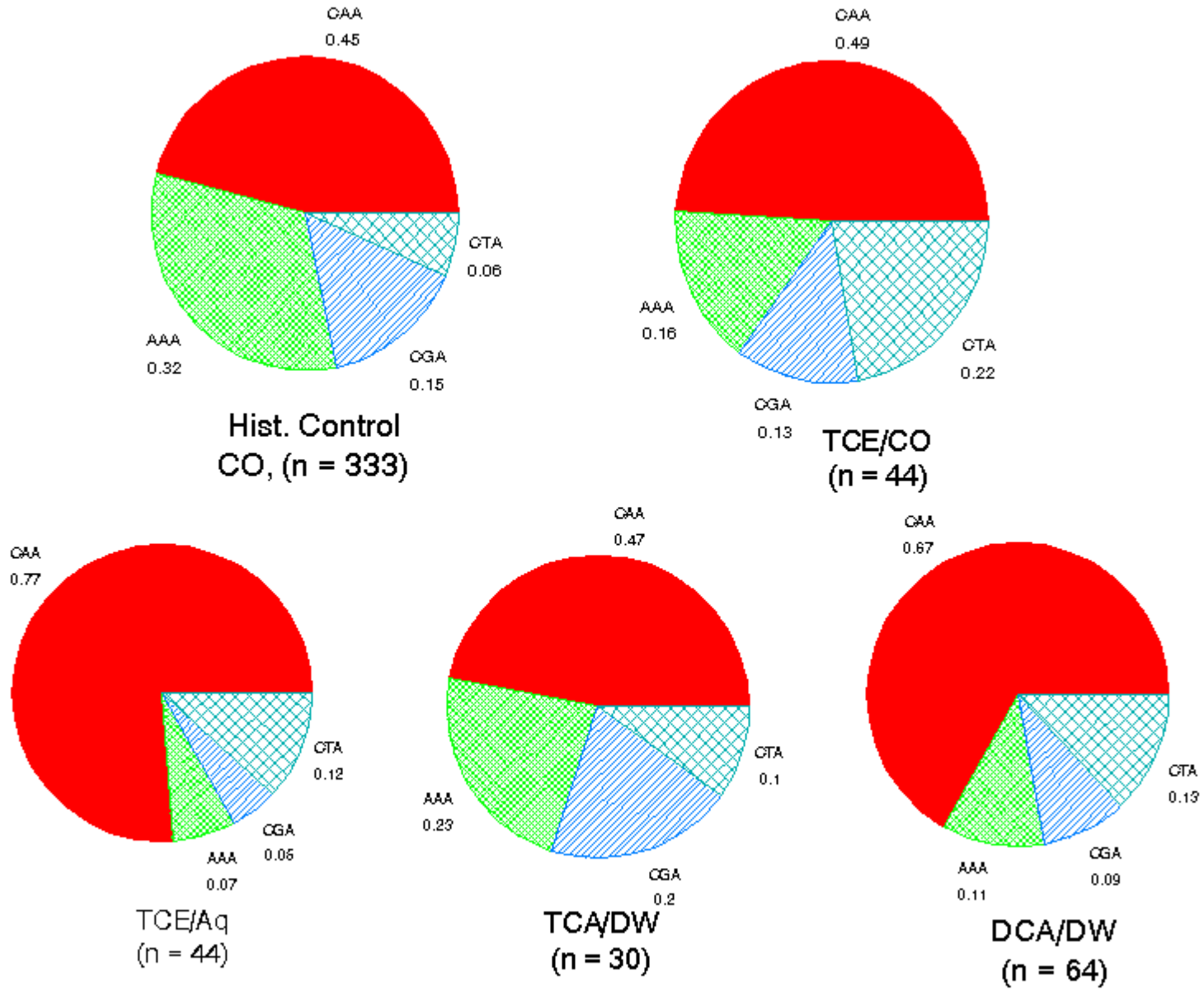

Figure 1. Mutation frequency and spectra codon-61 of H-ras in tumors taken from mice treated with trichloroethylene and its metabolites. Historical control values derived from reference 5. Other data is a summary of unpublished data of the authors

As can be seen in Figure 1, the mutation frequency found in codon-61 of H-ras in tumors taken from TCA animals is twice as high as that observed in tumors taken from DCA- treated mice at the same treatment duration. It is important to note that as time progresses, the mutation frequency at H-ras codon 61 in these tumors increases (data not shown). This strongly suggests that induction of mutations in this gene is not the mechanism by which DCA induces tumors, but one that arises with clonal expansion. The mutation spectra produced by TCA, DCA and TCE/Aq differ from one another. It is important not to over interpret these data. However, it is clear that the mutation frequencies seen in tumors induced by TCE administered in aqueous media is significantly different than that noted when it was administered in corn oil. Second, it is important that the mutation spectra with TCE in aqueous media is quite low and comparable to frequencies seen with other tumor promoters (5). Third the mutation frequency seen with TCA is significantly higher than that seen in tumors induced by TCE. The frequency observed with DCA more closely matches that of TCE, but the match is not perfect. The differences between TCE and TCA are significantly different from one another, while tat produced by TCE and DCA are not. However the frequency of spectra seen with TCA could not be differentiated statistically from DCA. The mutation spectras also differ. The main difference appears to be significantly less frequent occurrence of the AAA and CGA mutations with TCE and DCA relative to tumors from historical controls

At this point, the data still suggest some role for DCA in the induction of liver tumors by TCE. Does this matter? Are the modes of action of DCA and TCA sufficiently different that they require completely different treatment from a risk assessment standpoint? Does DCA act by initiating tumors or by affecting the growth rates of clones that have been spontaneously initiated in mice. This is not a trivial question since very high doses of DCA can be genotoxic (25) and accumulation of mutated cells the liver of the transgenic *lacI* mouse was observed with long-term treatment at high doses of DCA (26). Our own data had shown that chronic treatment of mice with DCA resulted in a tumor size distribution that was skewed towards small sizes relative to tumors induced by TCA (12). The continued appearance of small tumors throughout a chronic bioassay is generally taken as evidence that the chemical has some initiating activity. Can this response be explained by a mechanism that does not require DCA or one of its metabolites to directly induce mutation? This question has been addressed experimentally in two ways, one *in vivo* and the other *in vitro*.

The *in vivo* experiments involved the use of magnetic resonance imaging (MRI) to follow the growth rate of tumors produced by DCA while the animal was being actively treated vs. the rate observed when treatment was suspended. Figure 2 is an example of how rapidly tumors can be stimulated to grow when a mouse is treated with DCA. This particular animal was treated with single dose of a tumor initiator, vinyl carbamate (3 mg/kg), at 14 days of age and at weaning was placed on DCA at 2 g/L of its drinking water for a 24 week period. During the last two weeks of this treatment the tumor shown in the image was followed at 4 time points over a 14 day period. As can be seen the volume of this tumor increased by about 20-fold in this short interval.

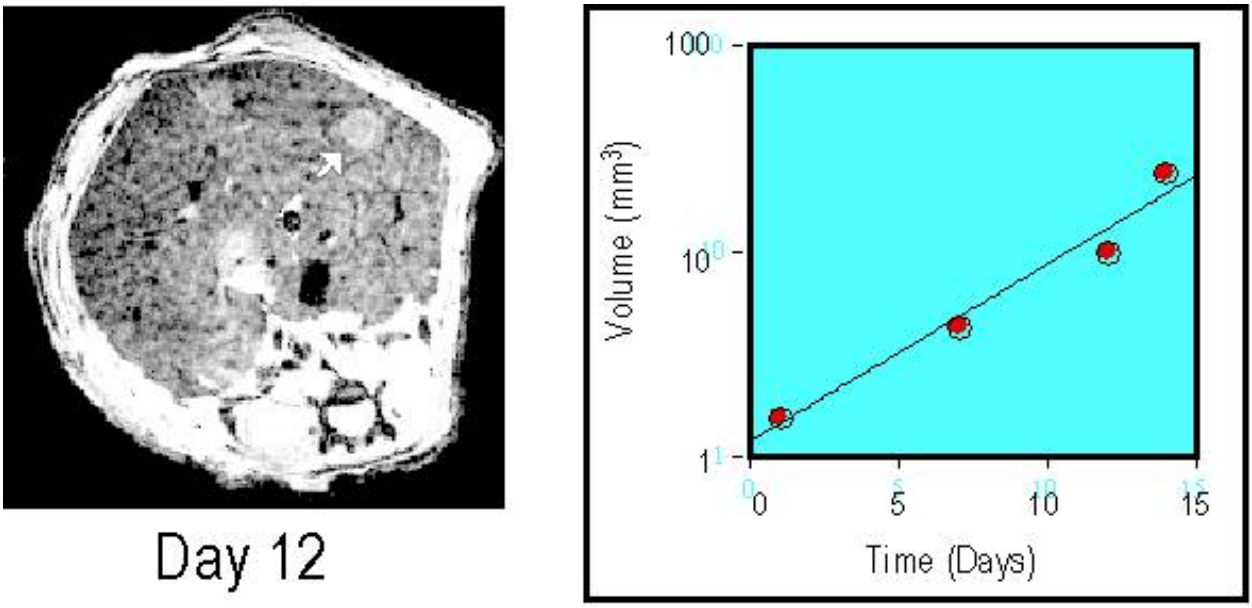

Figure 2. Measuring rates of tumor growth with magnetic resonance imaging. Tumor was produced by initiation with vinyl carbamate followed by administration of DCA at 2 g/L for approximately 24 weeks. The tumor was imaged on successive days indicated in the chart. Image provided was taken on day 12 of the experiment.

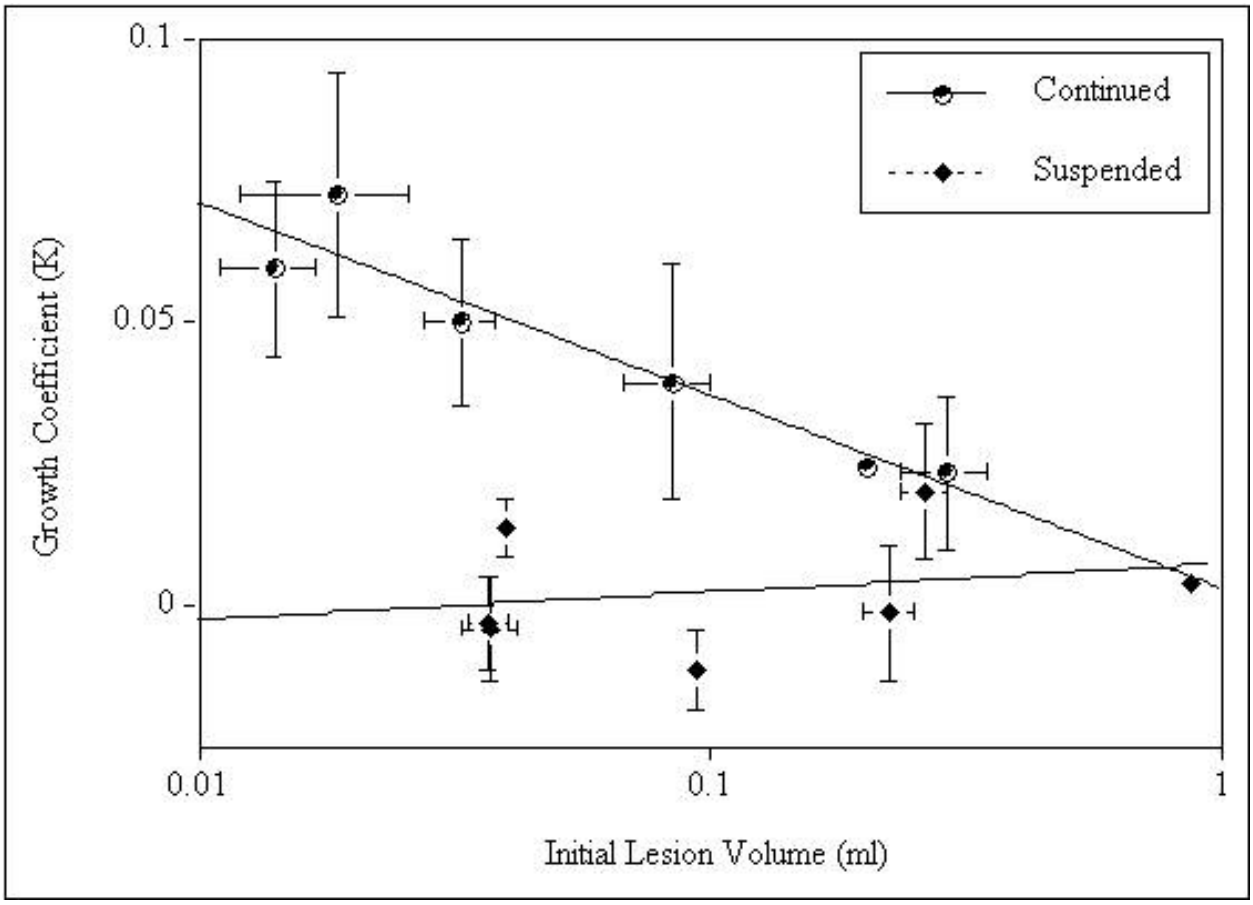

Figure 3. Effect of suspending DCA treatment (2 g/L) for two weeks on the growth rate of hyperplastic nodules in the liver of B6C3F1 mice. Each point represents the rate of growth of a single lesion between two successive MRI measurements 2-3 weeks apart. Horizontal bars represent the SD of lesion volume measurements while the vertical bars represent the corresponding SD for the growth rate measurement. From Miller et al. (13).

Figure 3 provides growth coefficients of 13 tumors that were observed in a group of 20 mice after 40 weeks of treatment at 2 g DCA/L of drinking water only (i.e. no tumor initiator was used). After the first detection of a measurable tumor, the mice were split into two groups containing tumors of matched size. The treatment of one group was suspended and over the next 2-3 weeks second images of the tumors were obtained. To minimize the time mice had to be anesthetized and restrained within the probe, a statistical method was used to obtain a best estimate of initial tumor size and final tumor sizes by analyzing the MRI images after the experiment was completed (13). This allowed images to be collected from a mouse within eight minutes.

Tumors in mice maintained on treatment continued to grow with coefficients that were inversely proportional to initial volume. However, tumors in the same size ranges in animals whose treatment had been suspended essentially stopped growing over the observation period. These data dramatically demonstrate that a major contribution of DCA towards tumorigenesis is its

effect on growth rates. These data also show that the effect of DCA is strongest on small tumors. If size is extrapolated back to the initiated cells, one can immediately see how the tremendous effects of DCA on growth could account a plethora of small lesions and the detection of mutated cells in clones whose growth has been so stimulated (see analysis in 13). The dose selected for these studies is important, because it is the dose at which DCA was previously shown to actually increase replication rates specifically in tumors (Stauber and Bull, 1997) and only doses higher than this result in a measurable increase in mutated cells in apparently normal liver. Therefore, the small lesions can be accounted for by postulating that initiated cells whose growth has been strongly stimulated to divide before the cell, presumably damaged, could be lost by apoptosis. Previous work by others has shown that even lower, tumorigenic doses of DCA suppress apoptosis (26). Therefore, we conclude that this mode of action can account for the apparent initiating activity of DCA.

The second approach involved more direct assessment of both DCA and TCA on growth of initiated cells into colonies of cells. Liver cell suspensions were prepared from the liver of B6C3F1 mice and these suspensions plated out on soft agar containing varying concentrations of DCA or TCA. By definition, only cells that can escape contact inhibition grow on soft agar. Consequently, cells that grow under these circumstances were already "initiated" for cancer in the animal in the absence of prior treatment. As illustrated in Figure 4, both compounds stimulated the growth of colonies on soft agar in a dose-dependent manner. Of more interest was the fact that the phenotype of the colonies that were stimulated to grow by the two chemicals were different, colonies stimulated by DCA stained positive for c-Jun, whereas those stimulated to grow by TCA were c-Jun negative (14). This was an exact match of the phenotypes of tumors that were produced *in vivo* by these two compounds.

It was of some concern that the dose-response curves for DCA and TCA were essentially identical in these studies. While the compounds have similar potency when administered in drinking water to animals, it was clear from pharmacokinetic studies that much lower concentrations of DCA were required *in vivo* than for TCA. Earlier work had shown that DCA sharply inhibited its own metabolism when administered in vivo. Consequently, we conducted a second experiment from animals that had been pretreated with 0.5 g/L DCA in drinking water for two weeks prior to isolating hepatocytes. The data obtained in this experiment demonstrated that pretreatment both 1) expanded the number of colonies that were sensitive to DCA treatment in vitro and 2) was active at much lower concentrations than required to stimulate growth of colonies from liver cells isolated from naive animals (14). Consequently, the effects observed *in vitro* appear to be consistent with in vivo observations in semi-quantitative as well as qualitative terms.

Research we are now conducting is attempting to define the mechanisms that are involved in the tumorigenic effects. We have yet to discover the direct effect of DCA that triggers the tumorigenic response. However, we have collected a body of data that strongly suggests that its tumorigenic effects may be related to, or at least parallel, its effects on intermediary metabolism.

A metabolic effect of DCA that seems to closely parallel the doses that cause cancer is a large accumulation of glycogen in the liver. Physiological control over glycogen synthesis and degradation in the liver is controlled by some of the same hormones that regulate cell division

and cell death. Consequently, we are exploring this phenomena in some depth. The initial results of this work have been described in detail by Kato-Weinstein et al. (22). Significant increases in hepatic glycogen content are first observed at 0.5 g/L, the lowest dose that has been shown to result in tumors and increases in a dose-related matter.. This result is of interest for several reasons. The most interesting is that tumors induced by DCA are uniformly glycogen-poor. It was also of interest that increased deposition of glycogen is a response to increased insulin secretion and that individuals with glycogen storage diseases are at high risk of developing hepatic tumors.

This observation has prompted us to examine the effects of DCA treatment on serum insulin concentrations and expression of insulin receptor normal liver and DCA-induced tumors. DCA treatment significantly reduces serum insulin concentrations over the same dose range in which glycogen was seen to accumulate. Moreover, these treatments cause insulin receptor concentrations in normal (i.e. non-tumor) liver cells to decrease sharply. However, if tumors are examined some months later, the tumors do not display this reduction in insulin receptor expression.

We have also looked to determine whether changes in a pathway dependent upon insulin (and other growth factors) is more active in tumor cells than in normal tissue from the same animal. The enzyme, mitogen-activated protein kinase, which is can increase cell replication rates and depress rates of apoptosis, is more heavily phosphorylated in tumor vs. non-tumor cells. The level of phosphorylation reflects activity of the pathway. However, these data do not establish that DCA is directly responsible for the increased phosphorylation, because such activation is a common property of liver tumors. However, the data are consistent with the basic idea that normal liver cells are able down-regulate insulin signaling and this does not appear to be the case with tumor cells.

DISCUSSION

Our research on the metabolites of TCE has confirmed that the induction of tumors can be accounted for entirely by DCA and TCA. Attributing a contribution from DCA to the overall response helps to resolve some of the inconsistencies in the genotype of tumors induced by TCA vs. TCE. However, the introduction of a contribution from DCA raised questions about TCE's overall mode action. Previous work had assumed that TCA was entirely responsible for the tumors that were induced. The mode of action assigned to TCA has been one of a peroxisome proliferator. Responses to peroxisome proliferators have been widely accepted as being of little concern to for human risk, particularly at the low doses that are likely to arise from environmental exposures. Therefore, a major goal of the project became the provision of a better description of DCA's mode of action.

Results of both the *in vivo* and *in vitro* work support the conclusion that the major effect of DCA is on the rate at which initiated cells undergo clonal expansion. Certainly at high doses, the effects of DCA *in vivo* appear to be largely, if not completely, accounted for by its stimulation of replication rates. At such high doses blood levels of DCA approach those required to produce mutation (22,25), however, there appears to be no need for such a mechanism. At lower, but yet tumorigenic doses of DCA, blood levels have been shown to be at least 3 orders of magnitude

lower than those producing mutations. Consequently, it is hard to invoke a significant role for mutation in the induction of tumors by DCA.

The demonstration that DCA modifies growth of previously initiated cells from mouse liver*, in vitro*, also indicate that these effects can occur at concentrations approaching those encountered when lower doses are administered *in vivo*. Significant increases in colony growth rates were measured from hepatocyte suspensions from pretreated mice at concentrations as low as 20 μM. This concentration is about five times higher than the effective blood concentrations seen *in vivo*. It is suspected that this discrepancy is accounted for by the still substantial rates of metabolism hepatocytes of mice even after inhibition of the glutathione S-transferase responsible for most of its metabolism (9,27). Finer estimates of effective concentration will require methods to measure small amounts of DCA in agar and to measure the rates at which it is metabolized in the assay. The *in vitro* segment of this experiment supports the basic hypothesis that DCA is causing clonal expansion *in vivo*, as well. Increased numbers of "initiated cells" were recovered as colony forming units from the livers of mice that had been subjected to pretreatment.

In summary, this research has shown that liver tumor induction by TCE can be accounted for by concerted action of two metabolites, DCA and TCA. In addition, the modes of action of both compounds are best described as one of stimulating clonal expansion. Consequently, liver tumor induction by TCE is more appropriately treated by a margin of exposure approach to risk assessment as mutation appears to play little, if any, role in the induction of liver tumors by this solvent. These data are currently under consideration in the Environmental Protection Agency's reevaluation of the cancer risk assessment for TCE.

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