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Project Title: **Mechanism Involved in Trichloroethylene-Induced Liver Cancer: Importance to Environmental Cleanup**

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Mechanisms Involved in Trichloroethylene-Induced Liver Cancer: Importance to Environmental Cleanup

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Research Objectives

The objective of this project is to develop critical data for improving risk-based cleanup standards for trichloroethylene (TCE).

Importance to DOE. Cleanup costs for chlorinated solvents found on DOE sites are most frequently driven by TCE because it is the most widespread contaminant and is generally present at the highest concentrations. Data that would permit increases in risk-based standards for TCE would reduce complex-wide cleanup costs by hundreds of millions of dollars.

Current Regulatory Actions that Research will Impact. EPA is currently reviewing its risk assessment for TCE. Richard J. Bull has worked with EPA on this review by writing the mode of action section of their determination. A presentation by James Cogliano of EPA at the 1999 Annual Society of Toxicology Meeting indicates that they have accepted the concept of nonlinear extrapolation for liver tumor induction by TCE. This project will end in FY 1999 with its major technical and policy objectives satisfied.

Problem Statement

One impetus for this research was data that indicated that TCE induced a shift in the mutation spectra observed in the H-ras codon 61 in mouse liver tumors. Such effects have been generally interpreted as indicating that the chemical is acting as a mutagen. As the project progressed, additional data were published that indicated that one metabolite of TCE, dichloroacetate (DCA), has mutagenic activity in vivo (Leavitt et al. 1997). Adaptations were made in the experimental design to address the importance of these effects to the induction of liver tumors by DCA. The results of this project argue strongly that selection (or promotion) is more likely responsible for these observations than are DCA-induced mutagenic effects.

The project is organized around two interrelated tasks:

Task 1 addresses the tumorigenic and dosimetry issues for the metabolites of TCE that produce liver cancer in mice, DCA, and trichloroacetate (TCA). Early work had suggested that TCA was primarily responsible for TCE-induced liver tumors, but several more mechanistic observations suggest that DCA may play a prominent role. This task is aimed at determining the basis for the selection hypothesis and seeks to prove that this mode of action is responsible for TCE-induced tumors. This project will supply the basic dose-response data from which low-dose extrapolations would be made.

Task 2 seeks specific evidence that TCA and DCA are capable of promoting the growth of spontaneously initiated cells from mouse liver in vitro. The data provide the clearest evidence that both metabolites act by a mechanism of selection rather than mutation. These data are necessary to select between linear (i.e., no threshold) and nonlinear low-dose extrapolation models.

Research Progress

Task 1 - Tumorigenesis Studies. Biologically based models for assessing health risk from TCE exposure requires that the metabolites responsible be known and the likely mode of action by which they act identified. Classically, TCA was considered the active metabolite in inducing liver cancer with TCE. However, a less abundant metabolite, DCA, received considerable focus in the past five years because the incidence and spectra of H-ras mutations are similar in tumors induced by DCA and TCE (Anna et al. 1994). Data on TCA were too sparse at the time to make a judgment.

We have now sequenced codon 61 of the H-ras protooncogene in a total of 30 TCA-induced and 64 DCA-induced liver tumors. In the past year, we sacrificed 47 mice that had been treated with 1 g/kg TCE in an aqueous emulsion (Alkamuls®) by gavage for 79 weeks. The aqueous vehicle was considered important because all the mechanistic work with the metabolites had been done using water vehicles. Corn oil has effects that might modify responses to a peroxisome proliferator like TCA. Forty-four tumors were recovered from these mice. This response rate was greater than that observed in lifetime studies done by the National Toxicology Program that used corn oil as the vehicle. The mutation frequency and spectra from tumors produced by TCE are compared with those produced by the two putative metabolites in Figure 1. Note that the period of exposure used in each experiment is identified at the top of the bar because data were presented last year that made it apparent that the mutation rate observed in DCA-induced tumors increased as the treatment period was extended. The TCA-induced tumors were examined at a single time point and dose (2 g/L for 52 weeks), whereas DCA-induced tumors were obtained at 0.5 and 2 g/L and at 52 and 87 weeks, respectively. Literature data (Maronpot et al. 1995) for the corn-oil-treated controls and TCE in corn oil groups were derived from mice treated for a full two years. Data generated in the present study show that there are substantial differences in the mutation frequency in H-ras codon 61 in tumors induced by DCA and TCA.

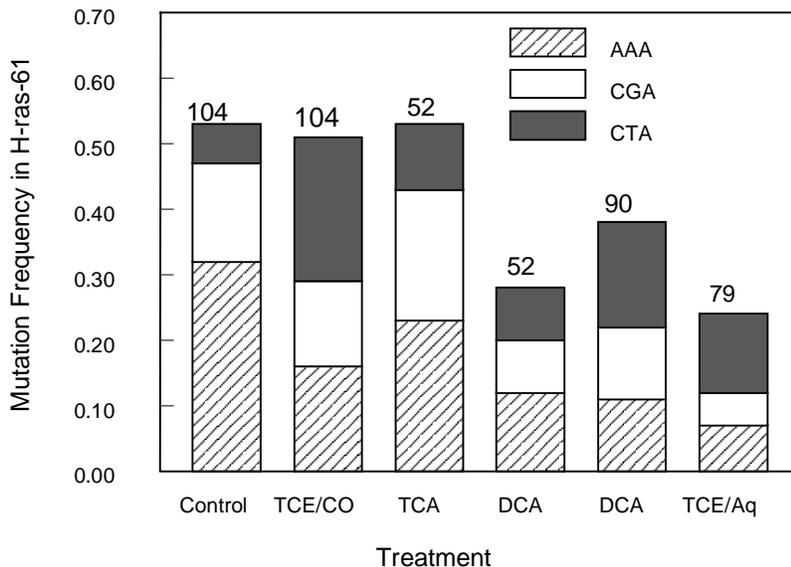


Figure 1. Mutation Frequency and Spectra of Codon 61 of the H-ras proto-oncogene in Liver Tumors of Mice Treated with TCE and its Metabolites. Historical control and TCE in corn oil (CO) groups are taken from Maronpot et al. (1995); remaining data from this study TCE/Aq indicate TCE was administered in an aqueous emulsion (Alkamuls).

The mutation frequency observed in control, TCE administered in corn oil, and TCA were all in excess of 50%. In contrast, the frequency in DCA-induced tumors and when TCE was administered in an aqueous vehicle are seen to approximate 25%. These data suggest that the high mutation spectrum previously reported for TCE (Anna et al. 1994) was influenced by the use of a corn oil vehicle.

The CTA mutation was observed in tumors induced by TCE in the aqueous vehicle at a higher fraction of total mutations observed than seen in historical control animals (Maronpot et al. 1995). This confirmed the nature of the spectra seen when TCE was administered with corn oil (Anna et al. 1994). DCA-induced tumors also appear to have a greater fraction of mutations at this site.

The lower mutation frequencies seen with DCA and TCE in the aqueous vehicle are much more consistent with previous observations made with other tumor promoters (Maronpot et al. 1995). Differences in the mutation spectra are not as convincing. If these data were expressed as the spectra of mutations within tumors carrying a H-ras mutation, as has conventionally been done, the results with DCA are more similar to that of TCE than that of TCA.

These data show that there are severe limitations in the use of mutation spectra as an indicator of the mode of action of a chemical carcinogen. Data presented below are much more consistent with the shifts in mutation spectra being produced by a selection process rather than compound-induced mutation. This is supported by the fact that, in tumors in which mutations were observed, the fraction of mutated DNA was always <50% of the DNA recovered from the lesion, strongly suggesting that H-ras mutations are late events in the development of mouse liver tumors. Clearly, relationships between mutation frequency and spectra are much more complicated than previously thought (Maronpot et al. 1995).

Because TCA has been shown to be a peroxisome proliferator, and that mechanism has already been discounted as having relevance for humans, much of our work has focused on DCA. We have been working with Kevin Minard and Robert Wind of the William R. Wiley Environmental Molecular Sciences Laboratory (EMSL) on imaging of tumors with magnetic imaging resonance (MRI) methods. An example of a high-resolution image of a mouse liver tumor is provided in [Figure 2](#). We used this capability to demonstrate that the major effect of DCA on tumor induction was its effect on growth rates. This was done by treating mice until small tumors were detected in a set of 15 animals. The mice were randomly assigned to two groups: one was maintained on DCA treatment and the other placed on distilled water for the next two to three weeks. At the end of the period, the same liver tumors were re-imaged. As shown in [Figure 3](#), tumors in those animals whose treatment was suspended stopped growing. This effect is most easily detected in smaller tumors because their fractional growth rate is much larger than that of larger tumors.

Using parameters obtained from the imaging of tumors, a model was developed to determine whether the effects of DCA on growth rates within tumors could explain the small lesion distribution. The model explicitly considered data previously developed on DCA's effects of replication rates in normal liver and tumors (Stauber and Bull 1997) and the observation of Snyder et al. (1997) that DCA suppressed apoptosis in normal hepatocytes. The combination of these two effects would provide a lesion distribution similar to that observed in mice that had been continuously treated with a genotoxic carcinogen. Not only do these data account for the lesion size distribution problem, they also explain the observation of Leavitt et al. (1997). These investigators found that increased numbers of *lac I* mutations were recovered in Big Blue transgenic mice treated with 3.5 g/L of DCA for 60 weeks but not at shorter time periods or at lower doses. These investigators were simply recovering cells from the mutations that

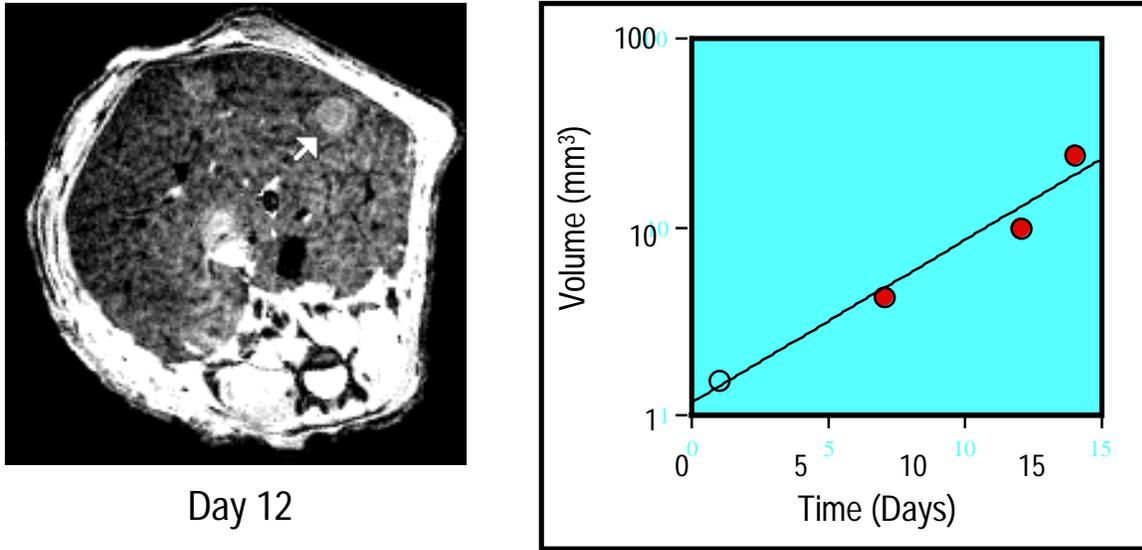


Figure 2. Measuring Rates of Tumor Growth with MRI. Tumor was produced by initiation with vinyl carbamate followed by administration of DCA at 2 g/L for approximately 24 weeks. The tumor was imaged on successive days indicated in the chart. Image provided was on day 12 of the experiment.

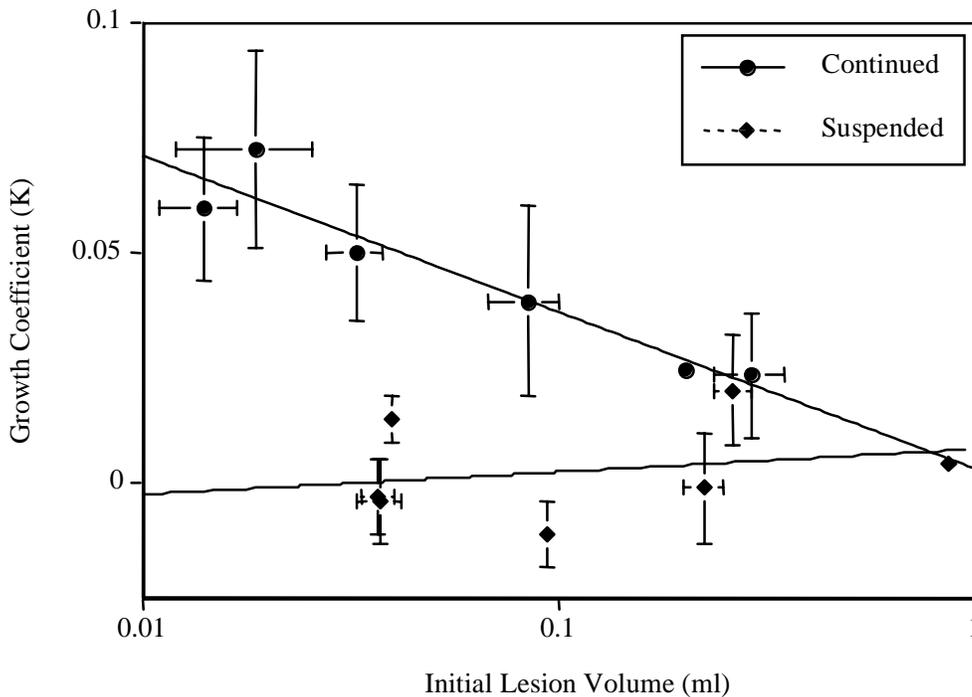


Figure 3. Effect of Suspending DCA Treatment (2 g/L) for Two Weeks on the Growth Rate of Hyperplastic Nodules in the Liver of B6C3F1 Mice. Each point represents the rate of growth of a single lesion between two successive MRI measurements 2-3 weeks apart. Horizontal represents the SD of lesion volume measurements, while the vertical bars represent the corresponding SD for the growth rate measurement.

were occurring in microscopic lesions, even though they had been careful to exclude tumor tissue from their samples. Therefore, these data add further support to the hypothesis that DCA is acting primarily, if not exclusively, as a tumor promoter.

Pharmacokinetic Results. Issues related to the dosimetry of the DCA that is produced from the metabolism of TCE have become much clearer in the past year. There are two issues: 1) the amount of DCA that is generated from a given dose of TCE and 2) the amount of DCA that must be in the liver to induce a carcinogenic response. As previously reported, DCA treatment results in substantial decreases in the metabolic clearance of the compound when mice are on chronic treatment at the high doses that have been used to induce cancer. Considerable confusion has been generated in the literature concerning the actual blood levels of DCA that result from the metabolism of TCE. Issues related to internal versus external doses of DCA were resolved in a paper published this last year (Kato-Weinstein et al. 1999). Blood concentrations resulting from treatment with 2 g DCA/L of drinking water are very high (approximately 500 μM) if measured during the period of water consumption (i.e., during the night in mice) and are less than 10 μM during the day. Lowering the concentration in drinking water by only 4-fold to 0.5 g/L resulted in peak concentrations of only 2-5 μM during the night, but which were immeasurable during the day (limit of quantitation = 1 μM). This is because the metabolism of DCA at these low doses is extremely rapid, in the range of 5-10 minutes. A concentration of 0.5 g/L of drinking water gives rise to an 80% tumor incidence in mice when administered for a lifetime; thus it indicates that low systemic concentrations of DCA are sufficient to induce cancer. Data from our initiation/ promotion studies indicates that 0.1 g DCA/L does not induce cancer. Concentrations of DCA seen in the blood from a dose of 1000 mg/kg TRI (the lowest carcinogenic dose tested by the National Toxicology Program) are maximally 1-2 μM . These data have brought the potential contribution of DCA to liver tumor induction by TCE to a much more reasonable level. A paper published last year (Merdink et al. 1998) demonstrated that the amounts of DCA produced in the metabolism of TCE, chloral hydrate, or TCA were very low. While there is evidence (i.e., the mutation frequency data) that DCA is contributing to the carcinogenic response, that contribution is shared with and fully accounted for by its effects in combination with TCA.

Task 2 - Establishing Selective Advantage as a Mode of Action for DCA and TCA. The most important aspect of this project was to show that the hepatocarcinogenic activity of TCE in mice is accounted for by a mode of action that does not involve direct interaction of the active metabolites with DNA.

The potency of a nongenotoxic tumor promoter depends on the ability of the compound to stimulate the survival and replication of initiated cells, and measuring this effect requires the actual isolation of initiated cells. A reliable characteristic of initiated cells is the ability to proliferate under anchorage-independent conditions, such as in culture over soft agar. Although growth over soft agar has been extensively studied using continuous cell lines (which are by definition already initiated), less is known about the response in primary cells.

The assay was used to measure the relative potency of DCA and TCA to promote the clonal growth of initiated hepatocytes (Stauber et al. 1998). As is shown in [Figure 4](#), incubation of hepatocytes from untreated mice with TCA or DCA caused a dose-dependent increase in anchorage-independent colonies that form over soft agar. Both DCA and TCA were approximately equally effective in promoting colony formation in vitro. In vitro, the effective concentration of either haloacetate to promote an increase in colony formation was between 200-500 μM . Furthermore, analysis of gene expression markers on DCA or TCA-promoted colonies indicated that the phenotype of these colonies reflect the phenotype of tumors promoted by chronic exposure in vivo (Stauber et al. 1998). Given the limited evidence for mutagenicity

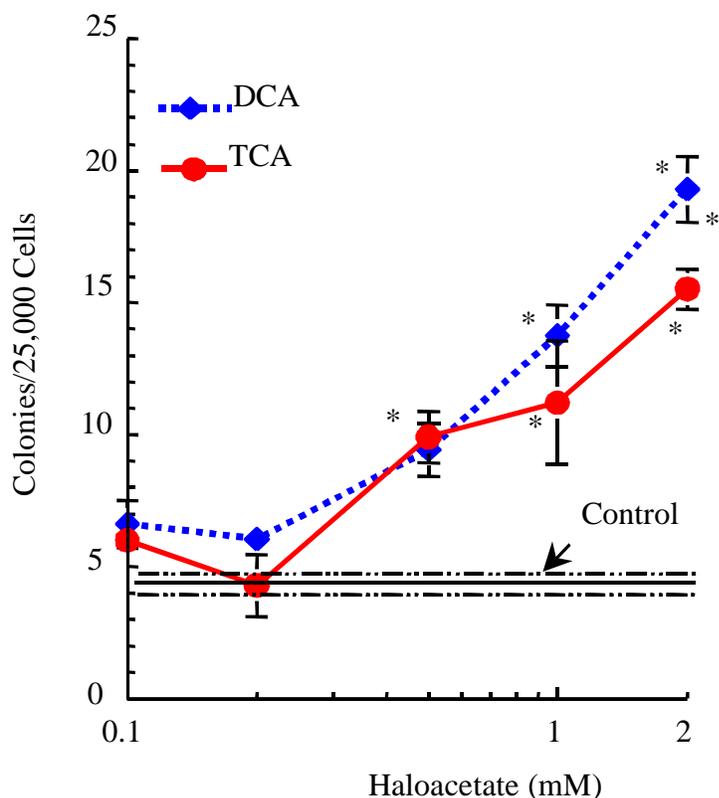


Figure 4. Effect of DCA and TCA on Anchorage-Independent Hepatocellular Colony Foundation in Vitro. Hepatocytes were isolated from untreated mice and cultured over agar in the presence of the DCA or TCA for 10 days. The number of anchorage-independent colonies was scored by microscopy.

*Differs from untreated control (p<0.05).

by these chemicals, these studies suggest that TCA and DCA promote the survival and growth of different populations of spontaneously initiated hepatocytes (Stauber et al. 1998), similar to previous reports for chronic in vivo exposure (Stauber and Bull 1997).

Although DCA and TCA exposures are equally potent in promoting anchorage-independent growth in vitro, comparison of these results with the kinetic analysis of potential DCA and TCA formation after TCE exposure raises questions about the role of DCA in tumors induced by TCE. At carcinogenic doses of TCE typically used in bioassays, sustained blood concentrations of $\geq 200 \mu\text{M}$ TCA are easily achieved. In contrast, the production of such concentrations of DCA in blood from metabolism of TCE is neither measurable nor predicted by kinetic models (Merdink et al. 1998).

Prior work has shown that DCA inhibits its own metabolism (Gonzalez-Leon et al. 1997). Therefore, the potency of DCA on stimulating colony formation was examined in hepatocytes taken from mice that were pretreated with a minimally carcinogenic concentration of DCA in drinking water (0.5 g/L). In hepatocytes isolated from DCA-pretreated mice an increase in colony formation above untreated controls was observed with concentrations of DCA as low as $20 \mu\text{M}$ in vitro (Figure 5). For comparison, concentrations $\geq 200 \mu\text{M}$ DCA were necessary to stimulate colony formation in hepatocytes from naïve mice, indicating that DCA pretreatment in vivo caused an increase in hepatocellular sensitivity by ≥ 10 -fold. These data largely rectify the discrepancies in the in vivo and in vitro potency of DCA.

Recent reports have associated increased serum insulin concentrations with occupational exposures to trichloroethylene (Goh et al. 1998). Increased amounts of insulin could also account for the large accumulations of glycogen in the liver of mice treated with DCA. However, our studies found that serum insulin levels were significantly depressed by DCA treatment. Figure 6 provides data obtained

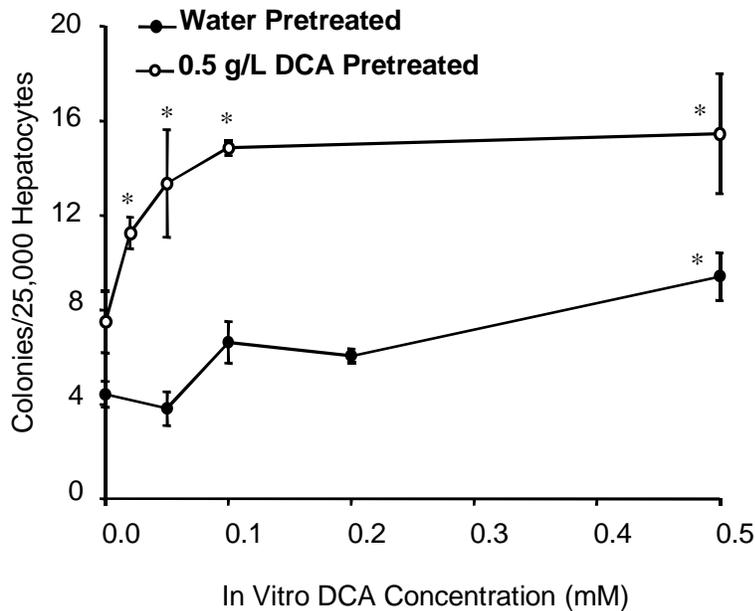


Figure 5. Effect of In Vivo DCA Pretreatment on the Sensitivity of Hepatocytes to Subsequent In Vitro DCA Exposure. Hepatocytes were isolated from mice pretreated with either water or 0.5 g/L DCA in water for two weeks. The cells were then cultured over agar in the presence of 0-0.5 mM DCA for 10 days and anchorage-independent colonies were scored.

*Differs from controls lacking DCA in vitro ($p < 0.05$).

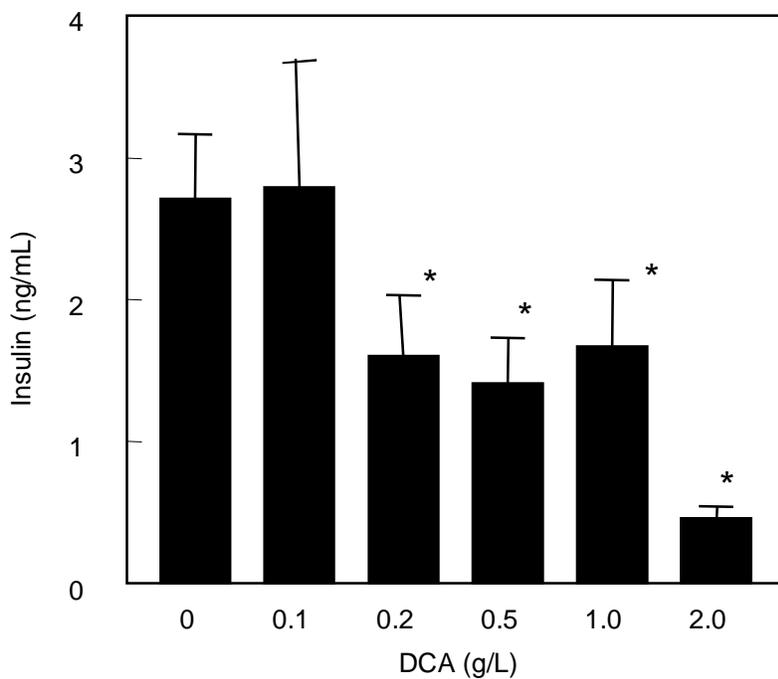


Figure 6. Serum Insulin Concentrations in B6C3F1 Mice that Have Been Treated with the Indicated Concentrations of DCA in Their Drinking Water for 10 Weeks, Measured at 3 AM; vertical bars indicate SEM of not less than six mice at each treatment level.

from animals on DCA treatment at 3 AM. This corresponds to the time when the mice are drinking water actively, when blood levels of DCA are at their highest, and during the active feeding period of mice. TCA did not have consistent effects on serum insulin concentrations (data not shown).

Parallel to the decreases in serum insulin is a decrease in the insulin receptor in the liver. Concentrations of DCA in drinking water that produce cancer cause substantial decreases in insulin-receptor expression after 10 weeks of treatment (Figure 7). Measurements of insulin receptor expression in normal liver and tumors in mice treated with DCA for 52 weeks displayed an interesting pattern (Figure 8). Insulin receptor in normal hepatocytes of treated mice again depressed (not as dramatically

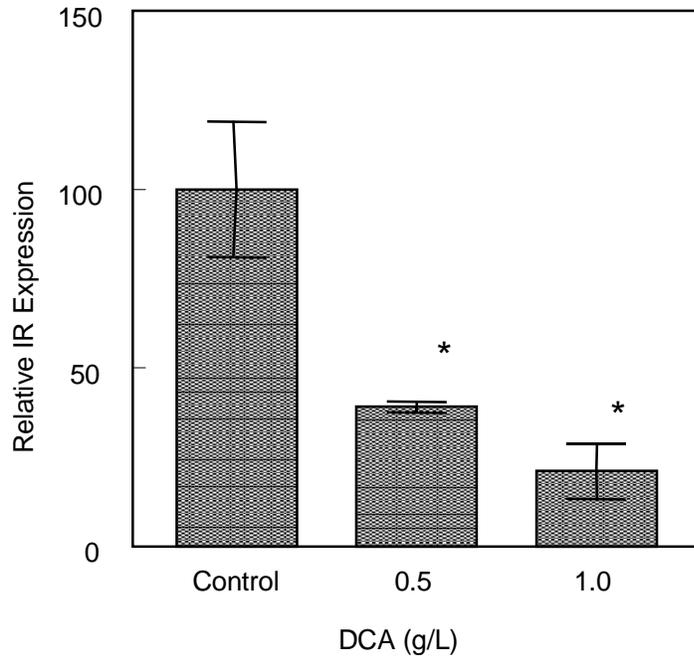


Figure 7. Insulin Receptor Expression in the Liver of Male B6C3F1 Mice Treated with the Indicated Concentrations of DCA in Drinking Water for 10 Weeks. Mice were sacrificed at 3 AM. Vertical bars indicate the \pm SEM of not less than six mice at each treatment.

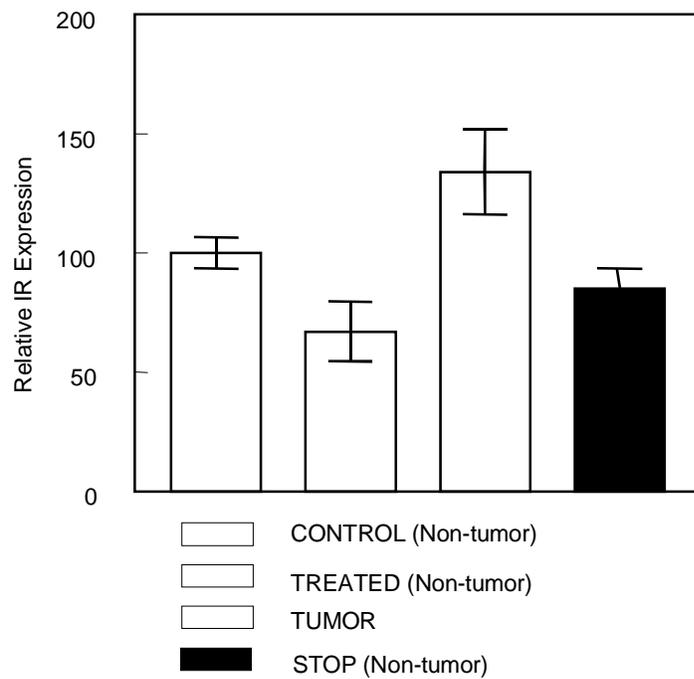


Figure 8. Insulin Receptor Expression in Hepatocellular Tumors of B6C3F1 Mice Treated with DCA (2 g/L) Relative to Surrounding Normal Tissue. Note that non-tumor tissue levels recovered to the point of control levels of IR expression when treatment was suspended for two weeks. Vertical bars indicate \pm SEM for not less than six animals or tumors.

as that observed at 10 weeks, but mice were sacrificed during the day rather than at night). In contrast, the expression of insulin receptor in tumors was twice that of surrounding tissues in DCA-treated mice. An interesting additional finding was that suspension of treatment for two weeks allowed insulin receptor expression to recover to control levels in the normal portions of the liver.

The insulin signaling pathway can influence carcinogenic effects by altering the rates of cell division and apoptosis (Tanaka and Wands 1996). In Figure 9, the levels of an active form of a downstream effector protein in liver from control animals, and normal liver and tumors from DCA-treated mice, are displayed. The activity of this protein (as measured by phosphorylation) was substantially increased in tumors. However, it is not activated by DCA treatment in the normal parts of the liver. These results are very compatible with the differential effects that DCA treatment has on replication rates in normal tissue and tumors. The depressed replication rates in normal tissue could be accounted for by the decrease in serum insulin concentrations, while the increased replication rates may be associated with the activation of the MAPK pathway. Increased sensitivity to insulin by increased insulin receptor levels could be one contributor to the increased activity of the MAPK and may contribute to the increased replication rates in tumors in DCA-treated mice.

Although there are effects of DCA that can be measured at the molecular level with chronic DCA treatment, these effects appear to have a latent period and are not reflected by *in vitro* treatment with DCA. This suggests that the mechanism by which DCA induces hepatic tumors may be indirect. DCA has been known for its ability to affect intermediary metabolism at high doses, primarily by inhibiting the pyruvate dehydrogenase kinase, resulting in accelerated Krebs' cycle activity. However, the apparent K_i for such effects is approximately 200 μM (Pratt and Roche 1979), whereas data provided in this study indicate that it can induce tumors at systemic concentrations two orders of magnitude lower. The only biochemical effect of DCA that occurs at these low concentrations in the blood is accumulation of glycogen (Kato-Weinstein et al. 1998). The dose-response relationship for glycogen accumulation in the liver parallels the low range of the tumorigenic doses (Figure 10). It is known that glycogen accumulation in DCA treatment apparently inhibits glycogen synthesis by a feedback mechanism. It is possible that this feedback is broader than previously appreciated, including the down regulation of insulin receptor expression and depressing serum insulin concentrations observed in this study. A characteristic common to all DCA-induced hyperplastic nodules and tumors is that they lack glycogen (Bull et al.

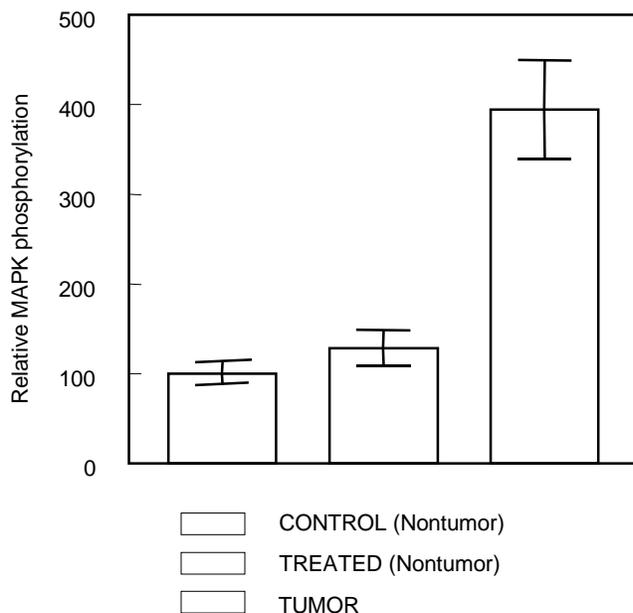


Figure 9. Mitogen-Activated Protein Kinase (MAPK) Phosphorylation Levels in Tumors and Non-Tumor Tissue in the Liver of B6C3F1 Mice Treated with DCA. Vertical bars indicate \pm SEM of tissue samples obtained from not less than six mice.

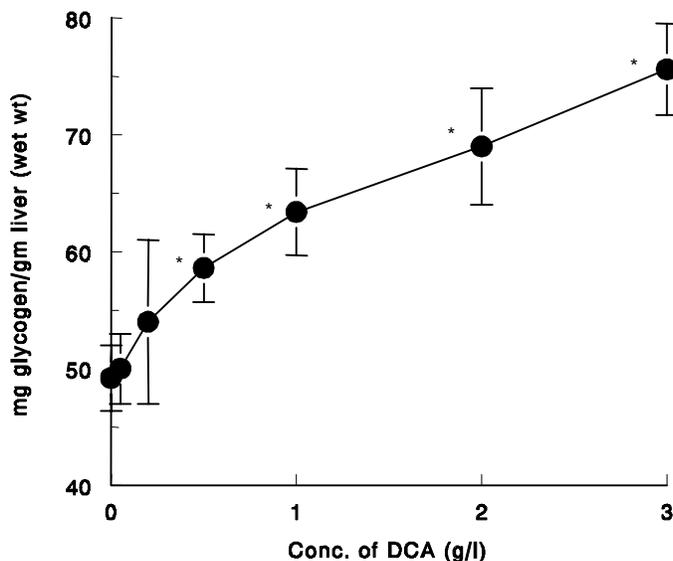


Figure 10. Liver Glycogen Concentrations in the Liver of B6C3F1 Mice Treated with Increasing Concentrations of DCA in Their Drinking Water for two Weeks. Each data point represents the mean of means obtained from not less than six mice + SEM.

1990). Others have demonstrated that this phenotype lacks glycogen synthase (Bannasch et al. 1984). This could also account for the fact that insulin receptor is not down-regulated, but appears up-regulated in DCA-induced tumors.

Implications for EM

This research has demonstrated that DCA and TCA can completely account for liver tumor induction in mice by TCE. The bulk of the experimental evidence suggests that neither TCE nor its two hepatocarcinogenic metabolites are mutagenic at concentrations that are relevant in vivo. Data in this project identify alternative modes of action for the two metabolites that are sufficient explanations for the carcinogenic response of TCE. These data have been accepted by the National Center for Environmental Assessment as a reasonable argument for a less than linear method of low-dose extrapolation.

The results from the colony formation assay clearly establish that these metabolites cause colony growth from initiated cells that spontaneously occur in the liver of B6C3F1 mice. In the case of DCA, a second mechanism, involving the release of insulin, occurs at a lower dose. However, this has not yet been shown to be responsible for liver tumor induction.

TCA carcinogenic activity is associated with its activity as a peroxisome proliferator. On principle, EPA has accepted that such compounds should be dealt with by the margin of exposure rather than the linear low-dose extrapolation. Peroxisome proliferation is of equivocal relevance as a tumorigenic mechanism in humans. In addition, TCA does not even induce liver tumors in rats.

The mechanism by which DCA produces liver cancer is still unclear, but it appears to be somewhat novel. It appears that one or more effects on intermediary metabolism creates alterations in insulin-signaling processes that may make a contribution to the carcinogenic responses. However, the key result of the present study has been to show that it clearly acts as a tumor promoter.

The following are critical data elements from this study:

- Both TCA and DCA stimulate the growth of anchorage-independent colonies from suspensions of hepatocytes derived from naïve mice that faithfully reflect the different phenotypes observed in vivo.
- DCA's effects on tumorigenesis was shown to directly affect the growth rate of tumors in vivo using MRI. This strongly supports the hypothesis that it is acting as a tumor promoter.
- Modeling of growth data obtained from MRI measurements indicates that previously reported effects of DCA on cell replication rates with hyperplastic nodules and suppressed rates of apoptosis can account for the lesion size distribution.
- These data also provide an explanation of the increased recovery of *lac I* mutant cells from mice chronically treated with DCA.
- The carcinogenicity of DCA in the liver appears to arise primarily as an indirect manifestation of its effects on intermediary metabolism.

A member of our research team (Richard J. Bull) is preparing the final draft on the mode of action paper being used by the EPA as a test case for their new Proposed Cancer Risk Assessment Guidelines. The arguments put forward above have been generally accepted by EPA and will form the basis for their decision. Because kidney tumors in rats are also produced with TCE, the impact of this work will be to change the potency estimate from that derived from liver tumor induction in the mouse to kidney tumor induction in mice. This should decrease the estimated risk per unit dose by approximately 10-fold. Informal discussion has been conducted with the Office of Water of the EPA. If the new guidelines are adopted in treating liver tumor induction by TCE, they intend to determine whether the drinking water MCL will need to be revised.

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