Final Report

Using Mode of Action to Assess Health Risks from Mixtures of Chemical/Physical Agents

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ABSTRACT

Interactions between carcinogens in mixtures found in the environment have been a concern for several decades. While interactions between initiators have received some attention, evaluations between tumor promoters with differing mechanisms of action have not been examined. In the present study, male B6C3Fl mice were used to study the responses to mixtures of dichloroacetate (DCA), trichloroacetate (TCA), and carbon tetrachloride (CT), each of which acts by a different mode of action. Mice were initiated by vinyl carbamate (VC), and then promoted by DCA, TCA, CT, or the pair-wised combinations of the three compounds. The effect of each treatment or treatment combination on tumor number/animal and tumor size was individually assessed.

Dose-related increases in tumor size were observed with 20 and 50 mg/kg CT, but each produced equal number of tumors at 36 weeks with the main distinction being a decrease in tumor latency at the higher dose. As the dose of CT was increased to > 100 mg/kg, substantial increases in the number of tumors per animal were observed, but the mean tumor size decreased dramatically. When administered alone in the drinking water at 0.1, 0.5, and 2 g/L, DCA increased both tumor number and tumor size in a dose-related manner. For TCA treatment at 2 g/L in drinking water, a maximum tumor number was reached by 24 weeks and was maintained until 36 weeks of treatment. Overall, TCA treatment produced dose-related increases in tumor number at 36 weeks of treatment. Thus, the lower doses of CT and TCA treatments apparently primarily affected tumor size rather than number.

Results with DCA were not as clear, as a true maximum tumor number was not clearly observed within the experimental period. Treatment of mice receiving a high dose of TCA (2 g/L of drinking water) combined with varying doses of DCA (0.1, 0.5 and 2 g/L) produced increased numbers of tumors at 24 weeks and 36 weeks. However, at 36 weeks of treatment, DCA produced a dose-related decrease in the size of tumors promoted by TCA. The low dose of TCA (0.1 g/L) decreased the number of tumors produced by a high dose of DCA; however, higher doses of TCA produced the same number as observed with DCA alone. Since these two chemicals produce lesions with differing phenotypes, the combination would have been assumed to be additive with respect to number, but this was obviously not the case. The inhibitory effect on number was not explained by differences in tumor size, although there was a tendency for a decrease in DCA-induced tumor size at the highest dose of TCA. DCA inhibited the growth rate of CT-induced tumors (CT dose = 50 mg/kg), with a tendency to increase numbers that were not statistically significant. On the other hand, TCA substantially increased the numbers of tumors observed early with CT -induced tumors, but this effect was not apparent at 36 weeks. This complex result was probably attributable to a coalescence of tumors based on the observation that the average tumor size in these groups was> 7 mm in diameter with a mean of 15 tumors per animal after 36 weeks of treatment. TCA administration in combination with CT actually caused a small reduction in tumor size at 36 weeks relative to CT alone. These data suggest that

the interactions between tumor promoters are dependent upon their modes of action and the cell types to which they provide a competitive advantage. Secondary modes of action can come into play as doses increase. In the present study, a secondary mode of action was most clearly observed as initiating activity of CT when doses exceeded 50 mg/kg.

1.0 INTRODUCTION

Mixtures of carcinogenic solvents are found in groundwater and soils at hazardous waste sites (Riley, 1992). While there is frequently data available for interactions between chemicals to judge risks from short-term exposures, data that describe how interactions influence the development of cancer are rare. This is largely because of the high cost associated with conducting complex interaction studies over the lifetime of experimental animals. The interactions between genotoxic and non-genotoxic carcinogens have long been recognized as having the potential of being synergistic as demonstrated in initiation/promotion studies. However, there are few systematic studies of interactions between chemicals considered to have non-genotoxic modes of action.

The specific case addressed in this study is the co-occurrence of chlorinated solvents at DoD and DOE facilities. The most common of these are trichloroethene (TCE), tetrachloroethene (PERC) and carbon tetrachloride (CT) (Riley, 1992). Two metabolites of TCE and PERC, dichloroacetate (DCA) and trichloroacetate (TCA), are entirely responsible for the liver cancer produced by these solvents (Bull, 2000). DCA and TCA are both tumor promoters in the liver, but their mechanisms differ. TCA is recognized as a peroxisome proliferator, whereas DCA, at low doses, appears to act through processes that control intermediary metabolism distinct from its ability to induce peroxisome synthesis at high doses. CT acts by a third mechanism, killing normal cells and encouraging the growth of resistant tumor cells by the reparative process that ensues (Dragani et al., 1986; Tanaka et al., 1987; Wada et al., 1990). Based on theoretical considerations, synergism could be expected when chemicals with differing mechanisms of action are involved in treatments. Evidence to date suggests that DCA and TCA act on distinct populations of tumor cells (Bull et al., 2002). If this is true, their effects should be no more than additive. It is assumed that CT acts non-specifically in stimulating the growth of different tumor cell types. Therefore, DCA and TCA should not add significantly to the numbers of tumors produced by CT at all doses that act strictly by tumor promoting mechanisms. Because of the diversity of mechanisms that appear to be involved, chemicals responsible for the liver carcinogenicity of chlorinated solvents appeared to be a good set with which to explore the limits on interaction between non-genotoxic carcinogens.

Underlying this research is the hope that classifying carcinogens by their modes of action will provide a simpler and more accurate means of predicting the hazards posed by a mixture over a range of exposure situations. If this is the case, knowledge about the dose-response characteristics of a particular mode of action at low doses should be applicable for estimating the risks associated with the combination. The advantage of this approach is that while the number of chemicals present in the mixture may be large, the number of modes of action responsible for the biological effects is small. Each mode of action may have many mechanisms that might contribute to changes in cell birth/death processes, but establishing mechanisms for every chemical would be very expensive. The modes of action represented by the three chemicals included are thought to be broadly representative in chemical carcinogenesis.

2.0 MATERIALS AND METHODS

2.1 Animals and Treatment

The experiments utilized male B6C3Fl mice initiated with 3 mg/kg vinyl carbamate (VC) at two weeks of age. These VC-initiated mice were then treated with DCA, TCA, CT, or in binary combinations of these chemicals for 18, 24, 30, and 36 weeks beginning at weaning (21 days of age). Treatment period refers to the period of time animals were subjected to DCA, TCA or CT (i.e., 0 time = date of weaning). DCA and TCA were administered in the drinking water at 0.0, 0.1, 0.5, and 2.0 g/L. These concentrations in drinking water lead to time weighted average intakes of about 20, 100, and 400 mg/kg (Bull et a1., 1990). The doses initially used in the study for CT were 50, 100 and 500 mg/kg administered by gavage in a 5% Alkamuls® in water vehicle based upon results in the bioassay conducted by NCI (1976). It became clear in the course of this study that 100 and 500 mg/kg were too high and the doses for CT were reduced for subsequent experiments to 0, 5, 20, and 50 mg/kg.

Table 1 lists the treatment combinations used in this study. The number of mice assigned to each experimental group was 10. It was necessary to divide the study into five segments for logistical reasons. Consequently, concurrent control animals were included for each of these segments (VC only). In the presentation and analyses of the data, these control groups were combined totaling 30 VC-only treated mice. Not shown in Table 1 are the untreated controls that were used as concurrent controls in all phases of the project that were not initiated. These data are not presented, as they played no role in the analysis (essentially only sporadically occurring tumors were observed in less than 1 % of the animals). Concurrent controls involving treatment with individual compounds (i.e., DCA, TCA, and CT) were utilized for cohorts of animals that were treated with combinations of these chemicals. Because of some significant differences in results obtained with groups treated in different time frames, the simultaneous controls have been used in the analysis and presentation of these data.

2.2 Measurements and Observations

Mice were sacrificed at 18, 24, 30, or 36 weeks of treatment. All treatment groups were represented in the 24 and 36 week sacrifices, except for animals treated with 100 and 500 mg CT/kg in which large tumor burdens required sacrifice at 30 weeks. At sacrifice, mice were weighed and the livers were removed and weighed. Livers were examined carefully; all lesions were identified and measured in two dimensions to the nearest 0.5 mm. The number of tumors and the size of each tumor were recorded for each mouse. The lesions with liver tissue were sliced and prepared in tissue cassettes. These samples were preserved in 10% neutral buffered formaldehyde (NBF) for 24 hours before being transferred to 70% ethanol. Randomly selected samples of these tissues were sectioned, stained with hematoxylin and eosin (H&E) and examined histologically to insure that the lesions observed were being properly classified as tumors (e.g., ranging from hyperplastic nodules to hepatocellular carcinomas).

2.3 Statistical Analyses

General linear regression and Poisson regression were used to analyze the effect of the chemicals and the time of the treatment on the tumor sizes and tumor counts respectively (Breslow and Day, 1980; Diggle, et al., 1994). For individual and pair-wise comparison of treatments, we analyzed the seven subsets (Table 2). The substance of the analysis was as follows: Let S_{ij} be the jth tumor size of the ith mouse; N_i be the tumor count of the ith mouse; DCA, TCA, and CT represent the effect of the corresponding chemicals on the tumor counts and size; WEEK represents the effect of time; and CT × WEEK, DCA × WEEK, TCA × WEEK represent the effect resulted from the interaction between the time and the chemicals. Therefore, for example, for subset 1, we can write the following equations:

$$S_{ij} = VC + CT + WEEK + (CT \times WEEK) \quad (1)$$

$$\log(N_i) = VC + CT + WEEK + (CT \times WEEK) \quad (2)$$

Where VC represents the background as well as VC effect.

SAS PROC GLM procedure were used to estimate the effect of CT, WEEK, CT*WEEK, and compute the p-values for equation (1). The PROC GENMOD procedure was used to estimate and compute the p-value of effects in equation (2). p-Values for the seven subsets are summarized in Table 2. For the last 4 subsets, the WEEKs considered were only 24 and 36 weeks for consistency of data comparisons.

A general linear model with random effects and a generalized linear model were applied to the tumor size data and tumor counts data respectively (Bailey, 1964; Tan 1991). Equations with quadratic terms of the concentrations of the chemicals, the time in weeks and the number of tumors in each mouse. It was found that the number of tumors and the size of the tumors were correlated. Therefore, tumor counts were modeled into tumor size data. Terms with a statistical significance level less than 5% were left in the model. Model fits were checked by examining the residual plots (the difference between the observed value and the predicted value by the linear model equations) and the correlation between residuals and the predicted values.

3.0 RESULTS

The complexity of the experiment precluded detailed histopathological diagnosis of tumors. To insure that lesions were being properly characterized as tumors, a sample of 100 lesions of varying size were submitted to a pathologist. Over 95% of the lesions examined were classified as nodules (nodular hyperplasia of the liver cells), hepatocellular adenomas (benign tumors), or hepatocellular carcinoma (malignant tumors). Tumors of mice receiving TCA were classified as carcinoma more often than tumors from mice receiving DCA.

The results of the statistical analyses for pairwise interactions and interactions in time are provided in Table 2. Because of the difficulty of visualizing the entire data set, these pair-wise interactions are presented in a series of figures designed to illustrate the important interactions that were identified. In some cases, this led to the depiction of control groups more than once. However, the statistical analyses presented relied upon the entire data set, not the individual groups as they are depicted in the figures. In these figures, the statistically significant interactions have been identified. The simple pairwise comparisons are summarized in Table 2.

3.1 Dose and time response with treatments by individual chemicals

Figures 1, 3, and 5 display the yield of tumors observed with dose, time, and the chemical used in treatment. Figures 2, 4 and 6 provide measures of tumor size. For simplicity, the results with CT have been limited to doses of 20, 50 and 100 mg/kg as lower doses (5 mg/kg) had no effect and the highest dose produced tumors that were too numerous to be counted accurately. All other data are displayed.

The character of the dose-response with respect to tumor yield with CT treatment became very non-linear as the doses administered were ≥100 mg/kg. The 50 mg/kg dose produced a response that reached a plateau of about 14 tumors/animal that was maintained between 24 and 36 weeks of treatment. The number of tumors observed with 20 mg/kg CT displayed an increased latency relative to that seen with the 50 mg/kg dose, but produced essentially the same number of tumors as observed with 50 mg/kg at 36 weeks. In contrast, the 100 mg/kg group produced approximately 40 tumors· per animal by 30 weeks with no sign of a plateau. At this time, the group had to be sacrificed as the animals were becoming morbid. The 500 mg/kg dose resulted in approximately 90 tumors/animal at this time (data not shown because accurate counts were not possible) and this group also had to be sacrificed. Figure 2 provides the data on tumor size. At 20 mg/kg per day, there was no difference in tumor size compared to the control group (VC-only). At 50 mg/kg, the mean tumor size increased steadily with time to a mean diameter of > 9 mm at 36 weeks. In contrast, the mean tumor size observed with 100 mg (Figure 2) and 500 mg/kg (not shown) did not increase with time of treatment beyond 24 weeks, remaining at less than 5 mm in mean diameter.

DCA treatment increased the numbers of tumors per animal with time and dose (Figure 3). An irregularity in the progression of tumor numbers appeared at 24 weeks and 30 weeks in the 2 g/L

treatment group. Since the data provided in this figure were collected from animals all initiated with VC at the same time, the anomaly cannot be attributed to differences in dose of initiator or to different shipments of animals. However, these results were not outside of the normal statistical expectations, so this apparent anomaly has been judged due to sampling variation. Given the anomaly, the increase in tumor yield was dose-dependent and statistically significant. There was no significant interaction between dose and time. The data indicate a more or less parallel response with time at 0.5 g/L and essentially no response at 0.1 g/L. The effect of DCA treatment on tumor size (Figure 4) shows progressively increasing size with time and an appropriate dose-response that does become significant even at the lowest dose at 36 weeks of treatment. However, it is important to point out that the tumor yield seen with the highest dose of DCA approximated that observed with 50 mg/kg CT and that the mean tumor size was less than 50% of that observed with this dose of CT.

TCA at 2 g/L of drinking water increases tumor numbers to a maximum at 24 weeks of treatment that is maintained through 36 weeks (Figure 5). At the lower doses, tumor numbers are increased significantly in a dose-related manner at 36 weeks. At 0.5 g/L TCA, the total number of tumors approaches the maximum established with the high dose of TCA at 36 weeks, but no earlier. TCA treatment increases tumor size with time of treatment in a dose-related manner (Figure 6). At all time points, the effect of TCA on tumor size is substantially greater than observed with DCA, but remains below that observed with 50 mg/kg CT. The interaction of the dose of TCA and time were not statistically significant. However, both dose and time contributed to the overall tumor response.

3.2 Responses to binary mixtures of tumor promoting regimens

Interactions between DCA and TCA were found to vary by combination, ratio of doses and with time. Low doses of DCA (0.1 and 0.5 g/L) significantly increased the numbers of tumors observed with 2 g/L TCA at the 24 week sacrifice between two and three-fold (Figure 7), but had virtually no effect on the number of tumors/mouse that were observed at 36 weeks. As a consequence, the overall contribution of DCA (considering both time periods) to the response was not significant. On the other hand, there was a dose-related decrease in the size of tumors produced by TCA with increasing doses of DCA that was particularly evident after 36 weeks of treatment (Table 8). Consequently, there was a significant interaction between dose and time for tumor size with this treatment combination.

Tumors promoted by 2 g/L DCA at 36 weeks were reduced significantly in numbers by the low dose of TCA, 0.1 g/L, but the response at the 2 g/L doses of both compounds was essentially identical to that seen with DCA alone (Figure 9). It should be noted that the 24 week result with DCA was strikingly different than that reported in Figure 3, but this data was included as it was the concurrent control for this block of experiments. This was due to an anomalously high response in the prior experiment at this time point as mentioned in the description of Figure 3. There appears to be a small decrease in the mean size of tumors produced by DCA by the highest dose of TCA, but this was not statistically significant.

The effects that varying doses of DCA and TCA had on the numbers and sizes of tumors produced by CT promotion (50 mg/kg) were quite different. DCA appeared to increase the number of tumors produced by CT, but this was not statistically significant (Figure 11). All three doses of DCA significantly reduced the mean tumor size promoted by 50 mg/kg CT after 36 weeks of treatment (Figure 12).

The high dose of TCA also increased the number of tumors observed early in the experiment (Figure 13), but the total number of tumors seen per animal at 36 weeks was not significantly different from control. Most surprising was that this result appears as a substantial decrease in the number of tumors/animal between 24 and 36 weeks of treatment with 2 g/L TCA. TCA also produced a small decrease the size of tumors promoted by CT; these did not seem to follow any particular pattern with dose (Figure 14).

4.0 DISCUSSION

These results indicate that the study interactions between tumor promoters can be quite complex. Rather strong interactions were observed between the three chemicals using rather simple metrics of tumor number and tumor size in a limited number of initiated animals per experimental group.

It is important to recognize that the descriptive data derived from these studies are not appropriately used in conventional risk assessment. Nevertheless, these data can be important in developing formal biologically based models for low doses of chemicals that act as tumor promoters. The best evidence to support this use in modeling is the fact that the general character of relative latency and tumor multiplicity seen in studies of DCA and TCA in uninitiated mice was faithfully reproduced in these experiments (Bull, 2000; Bull et al., 2002).

The data strongly suggest that the population of cells responsive to promotion was finite in these initiated animals, with the tumor response reaching a maximum with both doses of CT < 50 mg/kg (discussed more fully below). The same maximum response of around 10-15 tumors/mouse was observed with TCA. Although a plateau was not achieved in the tumor numbers promoted by DCA, the somewhat lesser, but statistically indistinguishable maximum response seen with DCA is consistent with this interpretation. However, when given to uninitiated animals, DCA produces more lesions with shorter treatment periods (Stauber and Bull, 1997). Consequently, the use of an initiator does change the relative proportion of cells responsive to the two chemicals substantially. As a result, the probability of tumor induction by TCA was enhanced to a greater extent than it was for DCA.

A most surprising finding of this study was a clear differentiation of the effects of low (50 mg/kg) doses of CT in comparison with high doses. The fact that essentially the same numbers of tumors were seen at 36 weeks at 20 and 50 mg/kg and the plateau in the response seen at the higher dose suggests that essentially all lesions initiated by VC with the ability to progress to a tumor were promoted by CT at these doses. Thus, 50 mg/kg CT per day acts primarily as a very effective tumor promoter with little evidence of initiation as indicated by increased numbers of tumors at the expense of tumor size.

When doses of CT exceed 50 mg/kg, the response involved very large changes in tumor numbers with time and sharply decreasing tumor sizes. Our interpretation of increasing tumor numbers, particularly when coupled with decreases in mean tumor size is suggestive of some tumor-initiating activity (Luebeck and Moolgavkar, 1991). Therefore, we conclude that high, but not low doses of CT possess significant tumor-initiating activity. Recent research has shown that *trans*-4-hydroxy-2-nonenal, the major by-product of lipid peroxidation forms adducts with DNA (Hu et al., 2002). Thus, it is probable that the higher doses of CT encourage a wide variety of secondary mechanisms that lead to inflammatory responses (Perez-Alvarez et al., 1993; de Ferreyra et al., 1995) which produced sufficient radicals to generate lipid peroxidation products

to damage DNA (Castro et al., 1996) and significantly increase the rate of tumor initiation. Several authors have shown carbon tetrachloride and other cytotoxic agents are by far more effective in increasing tumor yields than mitogenic agents (Ledda-Columbano et al., 1992), but the doses used in these studies were in part the result of doses that also caused initiation (500-2000 mg/kg). The present study demonstrates clearly that CT is much more effective and specific than the other agents in increasing tumor size if the doses are ≤50 mg/kg.

It was of interest that interactions of low doses of one tumor promoting agent acting by one mechanism on the maximum response of another were bounded by additivity. It was of interest that additivity characterized the effects of TCA and CT when they were administered together at their maximum promoting doses at 24 weeks. The loss in tumor numbers with continued treatment appears to be explained by coalescence of tumors. Therefore, the effects of these two promoters are apparently independent, with TCA apparently affecting a specialized group of initiated cells not efficiently promoted by CT. In all likelihood, this is attributable to the fact that TCA is a peroxisome proliferator, which is known to promote tumors in mice that have phenotypes distinct from those produced by other tumor promoters (Bull, 2000; Bull et al., 2002).

The interaction between DCA and CT was, if anything, less than additive. While increases in tumor number/animal was increased by all three doses of DCA over CT alone, in no case was the response statistically significant. This less than additive response may be partially explained by an apparent decrease in the growth rate of tumors promoted by CT by DCA.

Antagonism appears to characterize the interaction between DCA and TCA, particularly at high doses. As the doses of both agents approached their individual maximally effective doses when given individually, there was no sign of additivity, but only antagonism. When this occurred, it primarily involved a decrease in the rate of tumor growth. The fact that the interactions primarily affect growth, suggests that the conditions established by one promoter are not necessarily advantageous to all the initiated cells that are present. Like TCA, DCA induces a variety of changes in intermediary metabolism, but their effects are quite distinct in the fact that DCA has major effects on carbohydrate metabolism, whereas TCA and other peroxisome proliferators primarily affect lipid metabolism (Kato-Weinstein et al., 2001; Linghor et al., 2001).

An interaction of particular interest was the ability of small dose rates of DCA to greatly increase the tumor numbers produced early in the experiment with TCA and with little indication of such an effect at a later time point. In this case the tumor sizes were small < 5 mm), not too numerous (7-12 tumors per animal) and are not explained by coalescence. Rather, it appears that DCA sharply depresses the tumor growth rate in TCA-induced tumors. In separate experiments, we have found additivity between low doses of DCA and TCA without the use of tumor initiators (Bull et al., 2002). In these experiments, it was shown that whereas a c-Jun+ phenotype was stimulated by DCA only a c-Jun- phenotype was observed with TCA alone. In combination,

however, both phenotypes were apparent. Therefore, these two chemicals appear to stimulate the growth of different phenotypes of tumors in the liver of male B6C3F I mice. Such a conclusion is supported by the extensive work of Pereira and coworkers (e.g., Pereira and Phelps, 1996); Latendresse and Pereira, 1997). The antagonism seen between the two compounds is marked by an ability of DCA to suppress growth of TCA-promoted lesions in a dose-dependent manner. These data provide a convenient, if topical, explanation for why trichloroethylene tumors tend to have properties that appear as a mixture of those produced by DCA and TCA. Trichloroethylene metabolism mimics this condition very well by producing low systemic concentrations of DCA and high levels of TCA (Merdink et al., 1998).

The basis for the decreased numbers of early lesions produced when low doses of TCA were combined at high doses with DCA was not an expected result, but it appears to be highly significant. Such interactions may arise from subtle effects on cell signaling processes. For example, concentrations of TCA much below those that induce peroxisome proliferation activate the PI3K (B.D. Thrall, unpublished data). However, the tumors observed with the higher doses of TCA have the same distinct phenotype as those induced by other peroxisome proliferators (Bannasch et al., 1997).

Provided that more explicit understanding can be developed for the modes of action of individual tumor promoters, these data suggest that the induction of liver cancer from mixtures of solvents may have predictable outcomes. The major conclusion is that these interactions are generally no more than additive. It was most interesting to note that additivity was only observed when a low, but effective, dose of one agent was superimposed on a high dose of another. When given at high doses, the effects were generally no greater than observed with either agent alone. A low dose of TCA was clearly antagonistic to a high dose of DCA and if one considered that the combined effect of DCA and TCA should have been additive at high doses since they affect different tumor phenotypes, this antagonism carried throughout the dose response curve for TCA. Apparently, these interactions involve some subtle modification of effects by one chemical in cells responsive to the other chemical. Consequently, our findings do not argue that interactions will extend below the effective doses of either chemical.

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Table 1. Combination of Treatments Studied

Week	DCA (g/L)	TCA (g/L)	CT (mg/kg)	Week	DCA (g/L)	TCA (g/L)	CT (mg/kg)
18	0.0	0.0	0	24	2.0	0.1	0
18	0.0	0.0	50	24	2.0	0.5	0
18	0.0	2.0	500	24	2.0	2.0	0
18	0.0	2.0	0	30	0.0	0.0	0
18	0.0	2.0	50	30	0.0	0.0	20
18	0.0	0.0	500	30	0.0	0.0	50
18	2.0	0.0	0	30	0.0	0.0	100
18	2.0	0.0	50	30	0.0	0.0	500
18	2.0	0.0	500	30	0.0	2.0	0
24	0.0	0.0	0	30	0.0	2.0	50
24	0.0	0.0	5	30	0.0	2.0	500
24	0.0	0.0	20	30	2.0	0.0	0
24	0.0	0.0	50	30	2.0	0.0	50
24	0.0	0.0	100	30	2.0	0.0	500
24	0.0	0.0	500	36	0.0	0.0	0
24	0.0	0.1	0	36	0.0	0.0	5
24	0.0	0.1	50	36	0.0	0.0	20
24	0.0	0.1	500	36	0.0	0.0	50
24	0.0	0.5	0	36	0.0	0.1	0
24	0.0	0.5	50	36	0.0	0.1	50
24	0.0	0.5	500	36	0.0	0.5	0
24	0.0	2.0	0	36	0.0	0.5	50
24	0.0	2.0	50	36	0.0	2.0	0
24	0.0	2.0	500	36	0.0	2.0	50
24	0.1	0.0	0	36	0.1	0.0	0
24	0.1	0.0	50	36	0.1	0.0	50
24	0.1	0.0	500	36	0.1	2.0	0
24	0.1	2.0	0	36	0.5	0.0	0
24	0.5	0.0	0	36	0.5	0.0	50
24	0.5	0.0	50	36	0.5	2.0	0
24	0.5	0.0	500	36	2.0	0.0	0
24	0.5	2.0	0	36	2.0	0.0	50
24	2.0	0.0	0	36	2.0	0.1	0
24	2.0	0.0	50	36	2.0	0.5	0
24	2.0	0.0	500	36	2.0	2.0	0

Table 2. p-Values from statistical analyses of tumor size and numbers/animal (yield)

Groups for Comparison	Source	p-value (tumor size)	p-value (tumor yield)
VC, VC + CT 20, VC + CT 50, VC + CT 100	CT Week CT × week[a]	0.0001 0.0001 0.0001	0.0001 0.0001 0.0001
VC, VC + DCA 0.1, VC + DCA 0.5, VC + DCA 2	DCA Week DCA × week	0.0852 0.0001 0.7182	0.0042 0.0146 0.7771
VC, VC + TCA 0.1, VC + TCA 0.5, VC + TCA 2	TCA Week TCA × week	0.0003 0.0001 0.5208	0.0001 0.0001 0.3624
TCA 2, TCA + DCA 0.1, TCA + DCA 0.5, TCA + DCA 2	DCA Week DCA × week	0.0001 0.0001 0.0027	0.4013 0.0041 0.4863
DCA 2, DCA + TCA 0.1, DCA + TCA 0.5, DCA + TCA 2	TCA Week TCA × week	0.1946 0.0001 0.0691	0.0386 0.0001 0.8868
CT 50mg/kg, CT + DCA 0.1, CT + DCA 0.5, CT + DCA 2	DCA Week DCA × week	0.0001 0.0001 0.0006	0.2540 0.8794 0.8001
CT 50mg/kg, CT + TCA 0.1, CT + TCA 0.5, CT + TCA 2	TCA Week TCA × week	0.1475 0.0001 0.0001	0.1985 0.0374 0.8235

[a] Indicating the interaction between CT and the time of the CT treatment

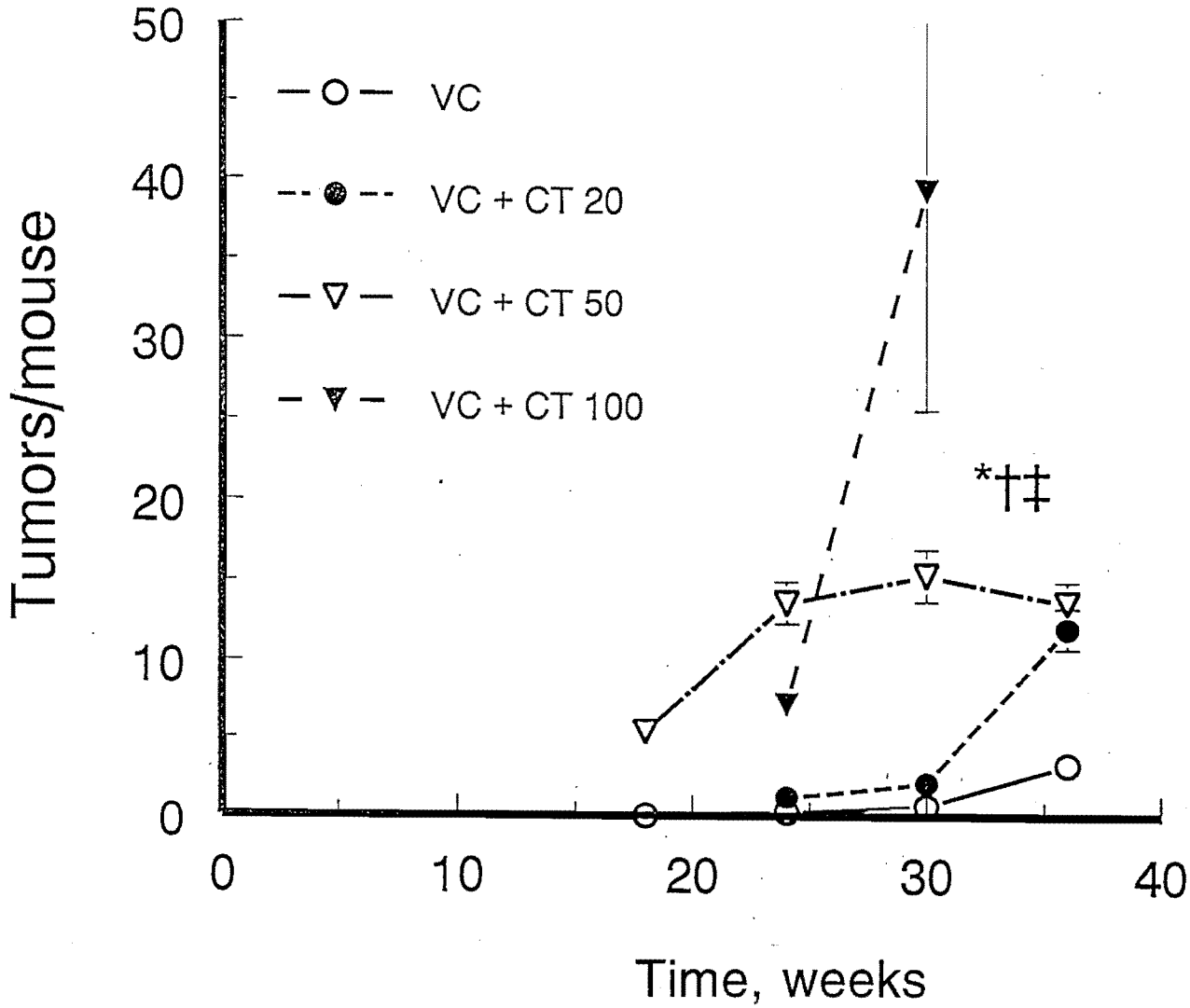

Figure 1. Tumors/Mouse in Carbon Tetrachloride (CT) Treated Mice. Tumor response of the liver produced in vinyl carbamate-initiated mice (3 mg/kg in 15-day old male B6C3F1 mice) treated with varying doses of CT from weaning until the sacrifice times indicated (Mean ±SEM). Symbols indicate that the response was significant ($P<0.05$) related to treatment (*), whether the effect was modified by week (†), or whether there were interactions between treatment and time (‡). See Table 2 for actual P values.

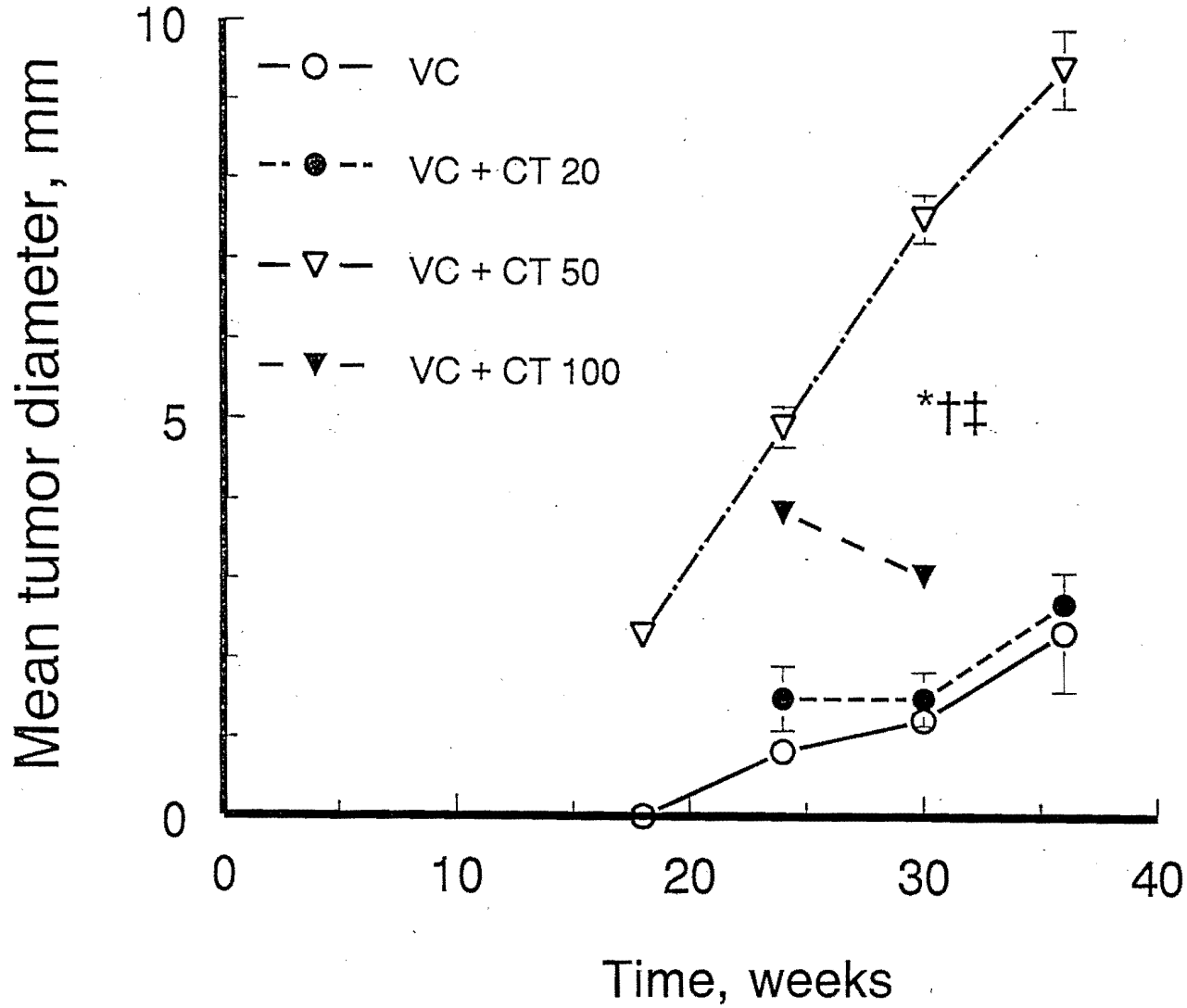

Figure 2. Tumor size in Carbon Tetrachloride (CT) Treated Mice. Tumor response of the liver produced in vinyl carbamate-initiated mice (3 mg/kg in 15-day old male B6C3F1 mice) treated with varying doses of CT from weaning until the sacrifice times indicated (Mean ±SEM). Symbols indicate that the response was significant ($P<0.05$) related to treatment (*), whether the effect was modified by week (†), or whether there were interactions between treatment and time (‡). See Table 2 for actual P values.

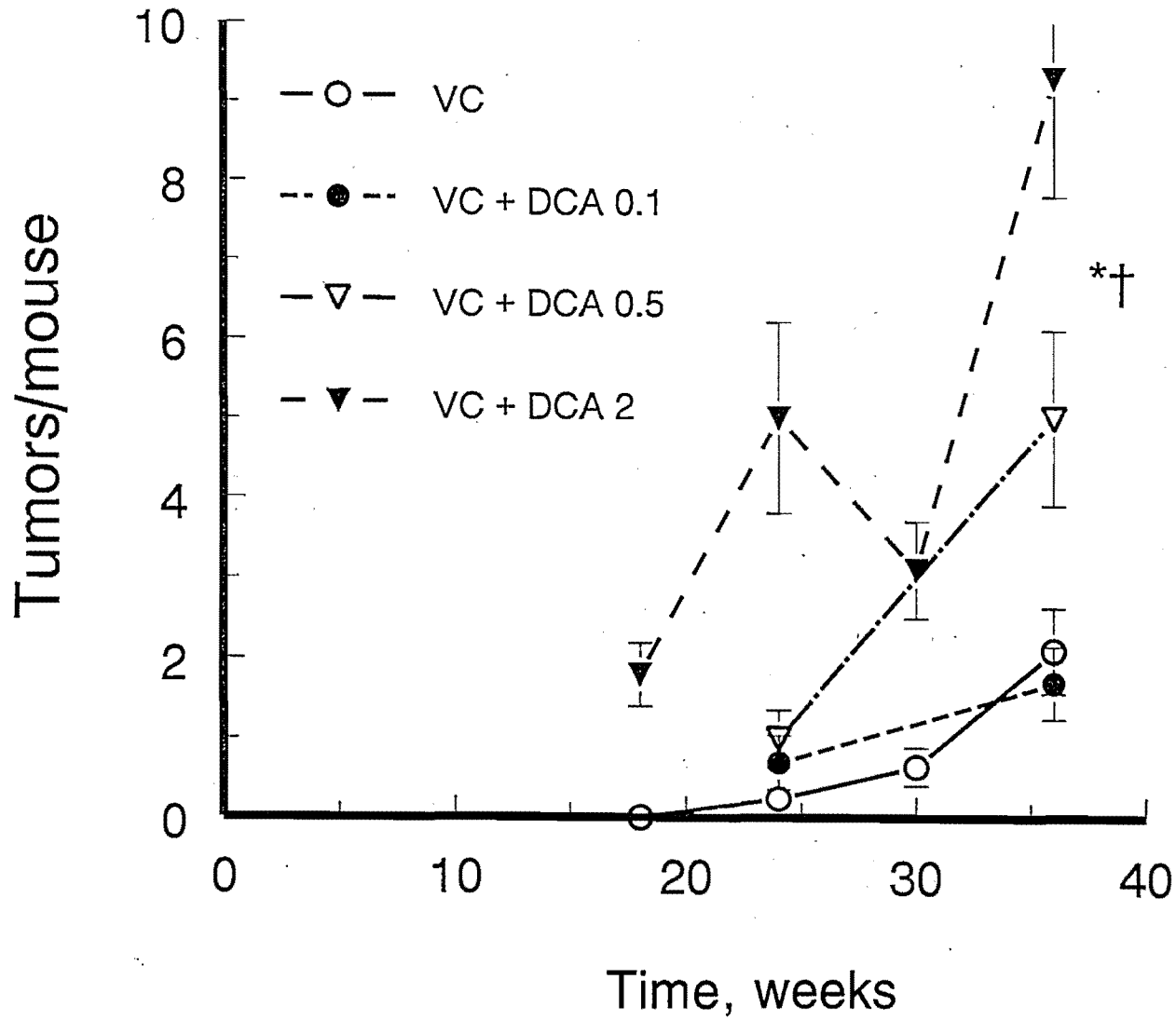

Figure 3. Tumors/Mouse in Dichloroacetate (DCA) Treated Mice. Tumor response of the liver of B6C3F1 mice initiated with 3 mg/kg vinyl carbamate at 15 days of age followed by treatment with DCA in drinking water at the indicated concentrations in g/L from weaning until the sacrifice times indicated. (Mean ± SEM) Symbols indicate that the response was significant ($P<0.05$) related to treatment (*), whether the effect was modified by week (†), or whether there were interactions between treatment and time (‡). See Table 2 for actual P values.

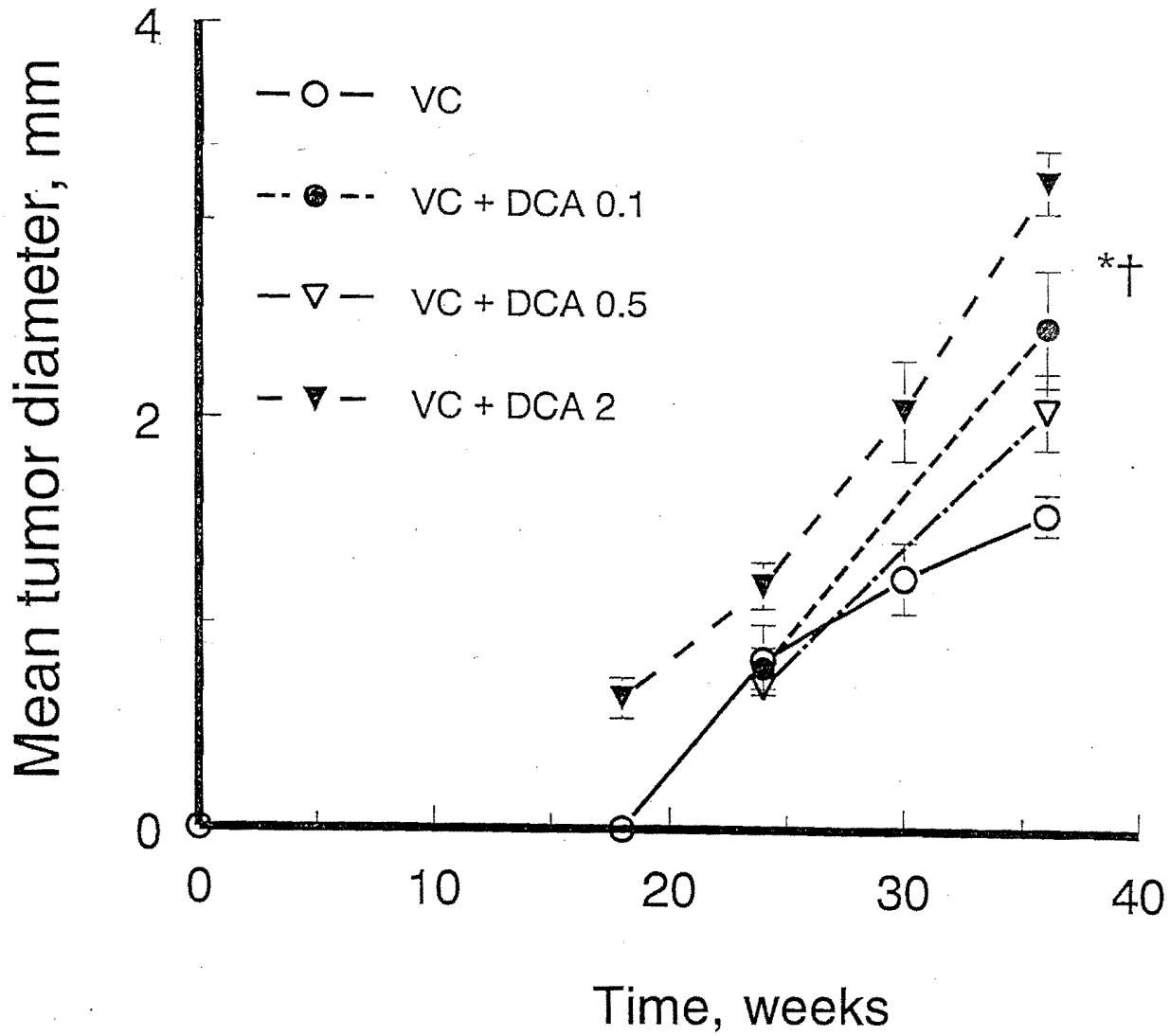

Figure 4. Tumor Size in Dichloroacetate (DCA) Treated Mice. Tumor response of the liver of B6C3F1 mice initiated with 3 mg/kg vinyl carbamate at 15 days of age followed by treatment with DCA in drinking water at the indicated concentrations in g/L weaning until the sacrifice times indicated. Symbols indicate that the response was significant ($P<0.05$) related to treatment (*), whether the effect was modified by week (†), or whether there were interactions between treatment and time (‡). See Table 2 for actual P values.

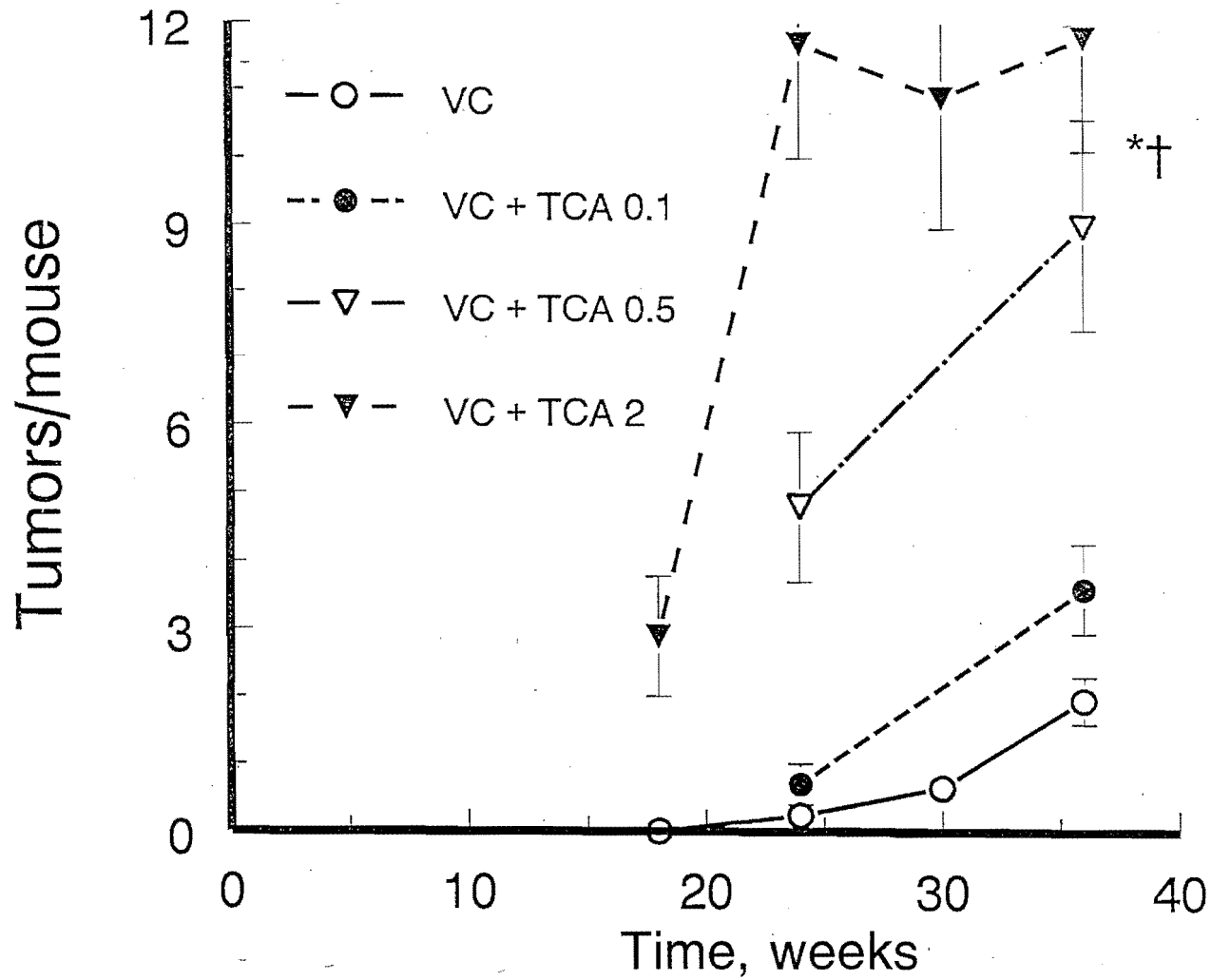

Figure 5. Tumors/Mouse in Trichloroacetate (TCA) Treated Mice. Tumor response in the liver of male B6C3F1 mice initiated with 3 mg/kg vinyl carbamate at 15 days of age followed by treatment with TCA in drinking water at the indicated concentrations in g/L from the time of weaning until sacrificed. (Mean ± SEM). Symbols indicate that the response was significant ($P<0.05$) related to treatment (*), whether the effect was modified by week (†), or whether there were interactions between treatment and time (‡). See Table 2 for actual P values.

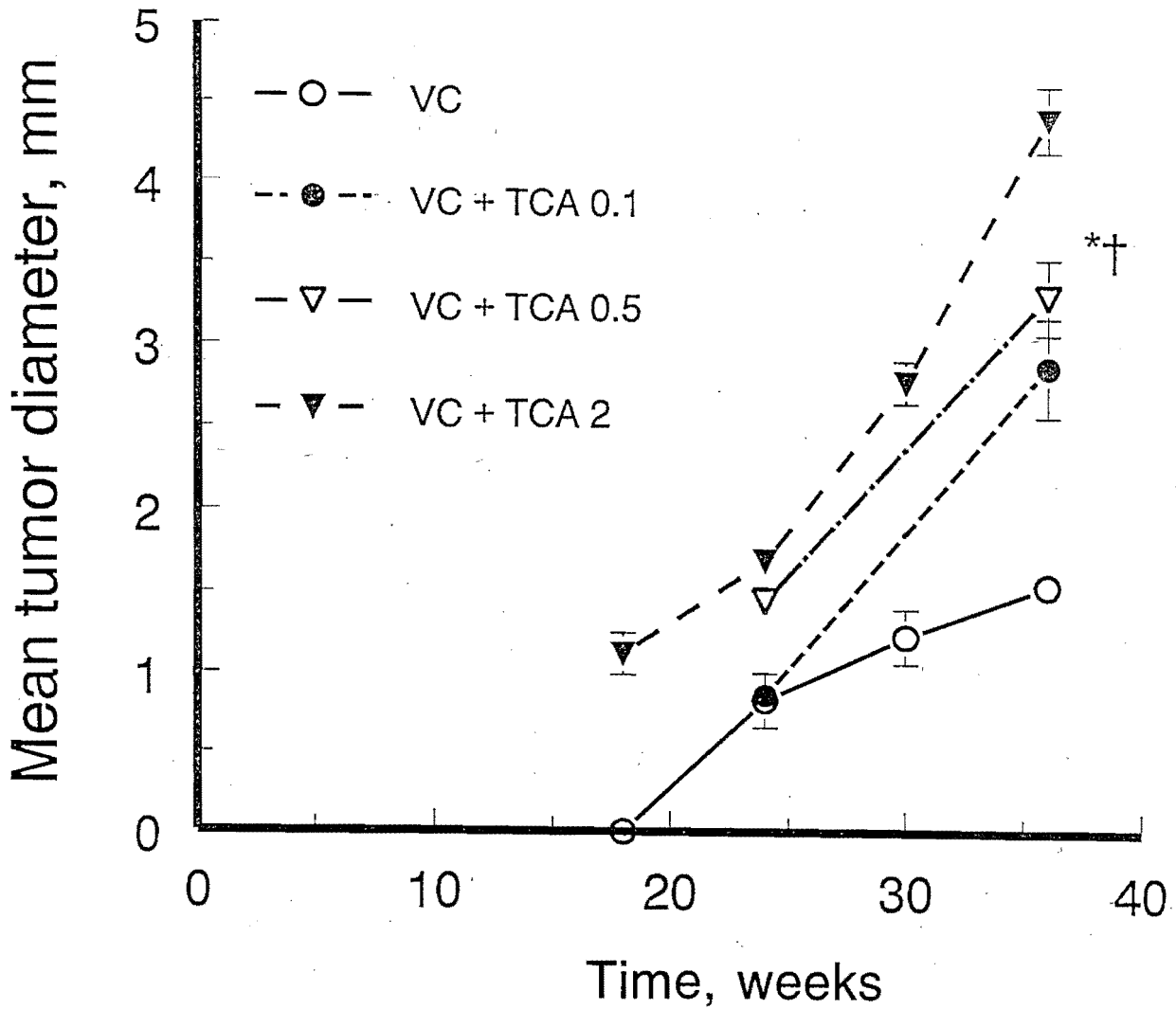

Figure 6. Tumors/Mouse in Trichloroacetate (TCA) Treated Mice. Tumor response in the liver of male B6C3F1 mice initiated with 3 mg/kg vinyl carbamate at 15 days of age followed by treatment with TCA in drinking water at the indicated concentrations in g/L from the time of weaning until sacrificed. (Mean ± SEM). Symbols indicate that the response was significant ($P<0.05$) related to treatment (*), whether the effect was modified by week (†), or whether there were interactions between treatment and time (‡). See Table 2 for actual P values.

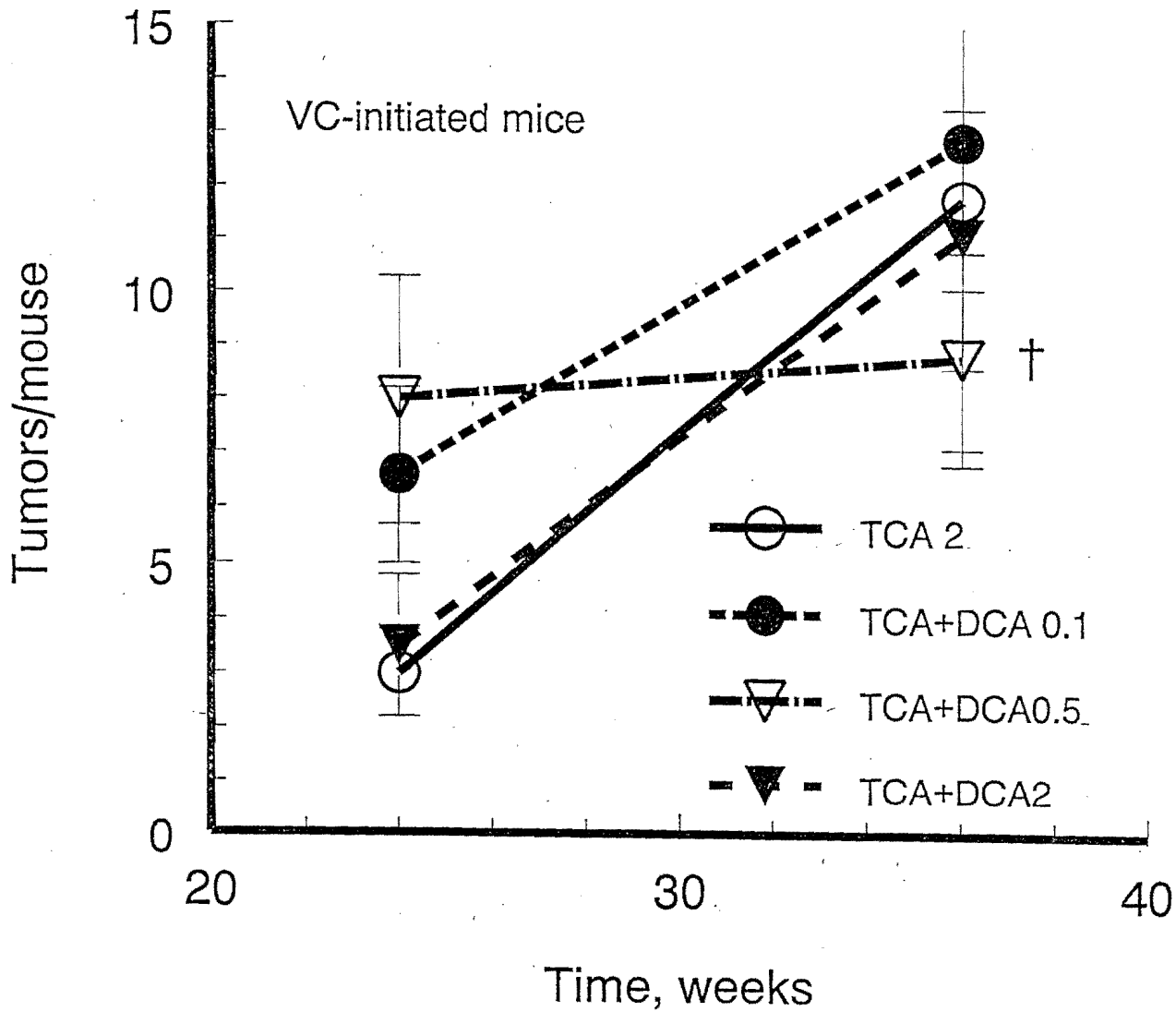

Figure 7. Tumors/Mouse in Mice Treated with a Single Concentration of TCA and Varying Concentrations of DCA. Tumor response in the liver of male B6C3F1 mice initiated with 3 mg/kg vinyl carbamate at 15 days of age followed by treatment with a single concentration of TCA in their drinking water (2 g/L) and varying concentrations of DCA ranging from 0 to 2 g/L. (Mean ± SEM). Symbols indicate that the response was significant ($P<0.05$) related to treatment (*), whether the effect was modified by week (†), or whether there were interactions between treatment and time (‡). See Table 2 for actual P values.

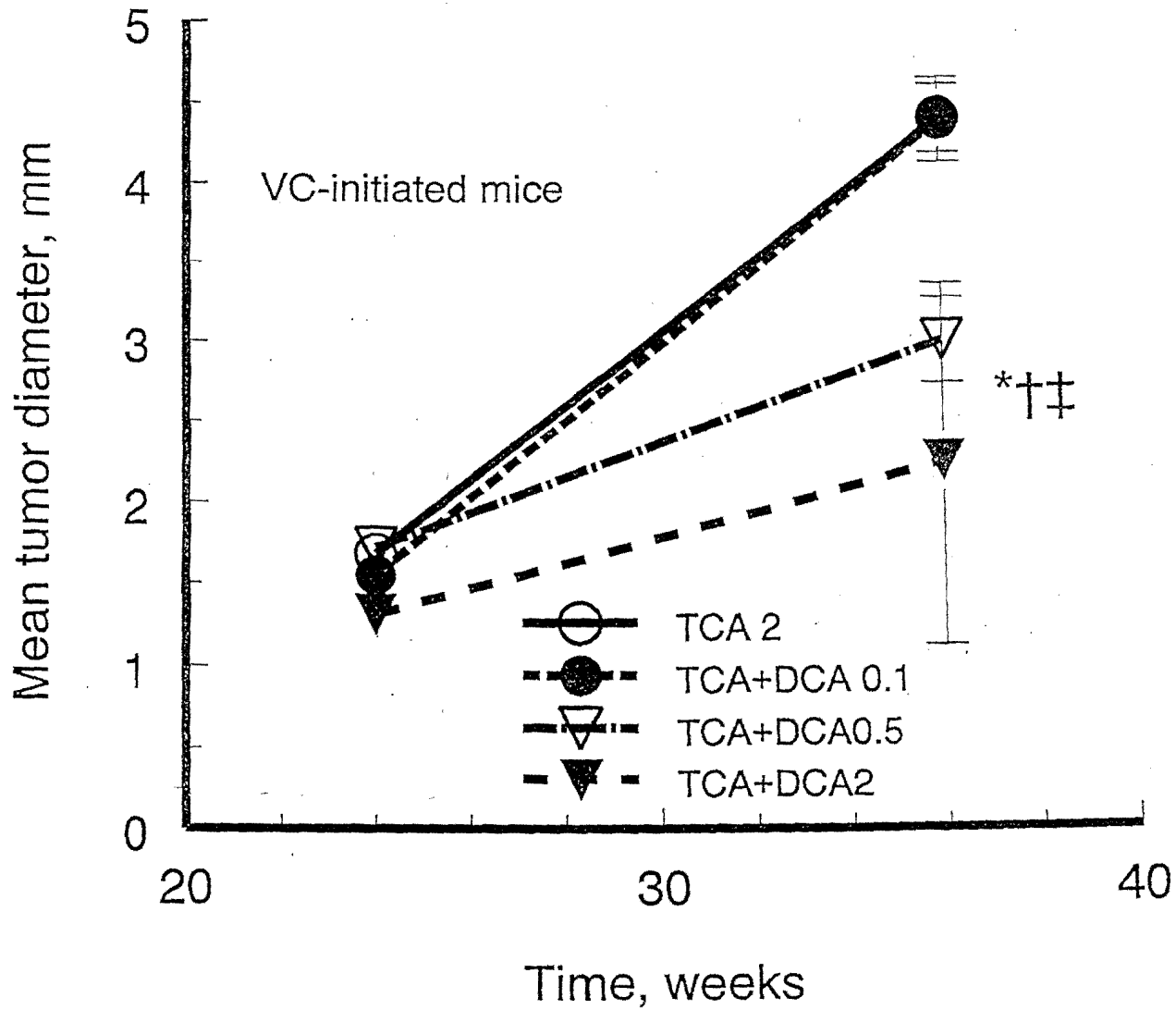

Figure 8. Tumor Size in Mice Treated with a Single Concentration of TCA and Varying Concentrations of DCA. Tumor response in the liver of male B6C3F1 mice initiated with 3 mg/kg vinyl carbamate at 15 days of age followed by treatment with a single concentration of TCA in their drinking water (2 g/L) and varying concentrations of DCA ranging from 0 to 2 g/L. (Mean ± SEM). Symbols indicate that the response was significant ($P<0.05$) related to treatment (*), whether the effect was modified by week (†), or whether there were interactions between treatment and time (‡). See Table 2 for actual P values

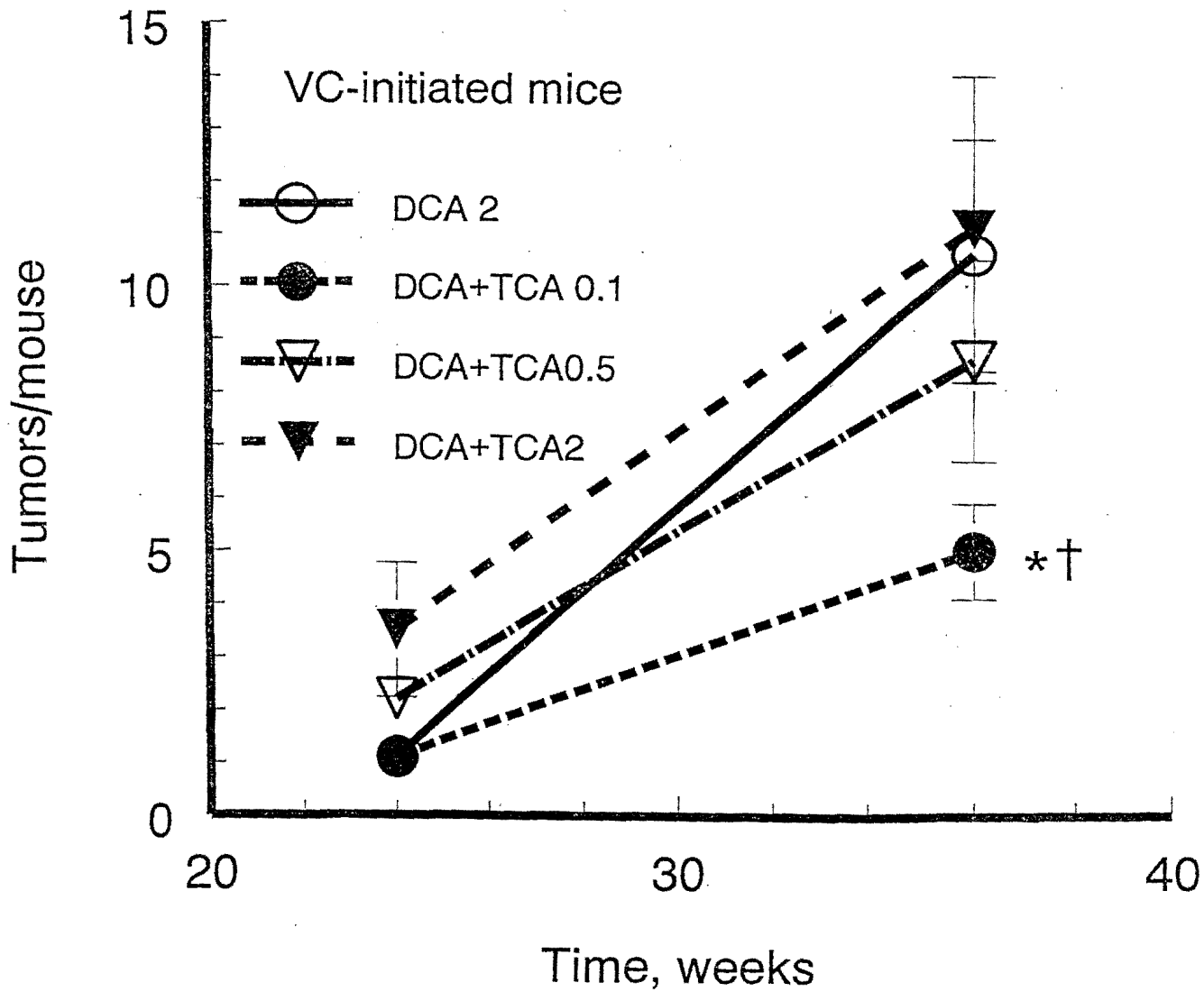

Figure 9. Tumors/Mouse in Mice Treated with a Single Concentration of DCA and Varing Concentrations of TCA. Tumor response in the liver of male B6C3F1 mice initiated with 3 mg/kg vinyl carbamate at 15 days of age followed by treatment with a single concentration of DCA in their drinking water (2 g/L) and varying concentrations of TCA ranging from 0 to 2 g/L. (Mean ± SEM). Symbols indicate that the response was significant ($P<0.05$) related to treatment (*), whether the effect was modified by week (†), or whether there were interactions between treatment and time (‡). See Table 2 for actual P values

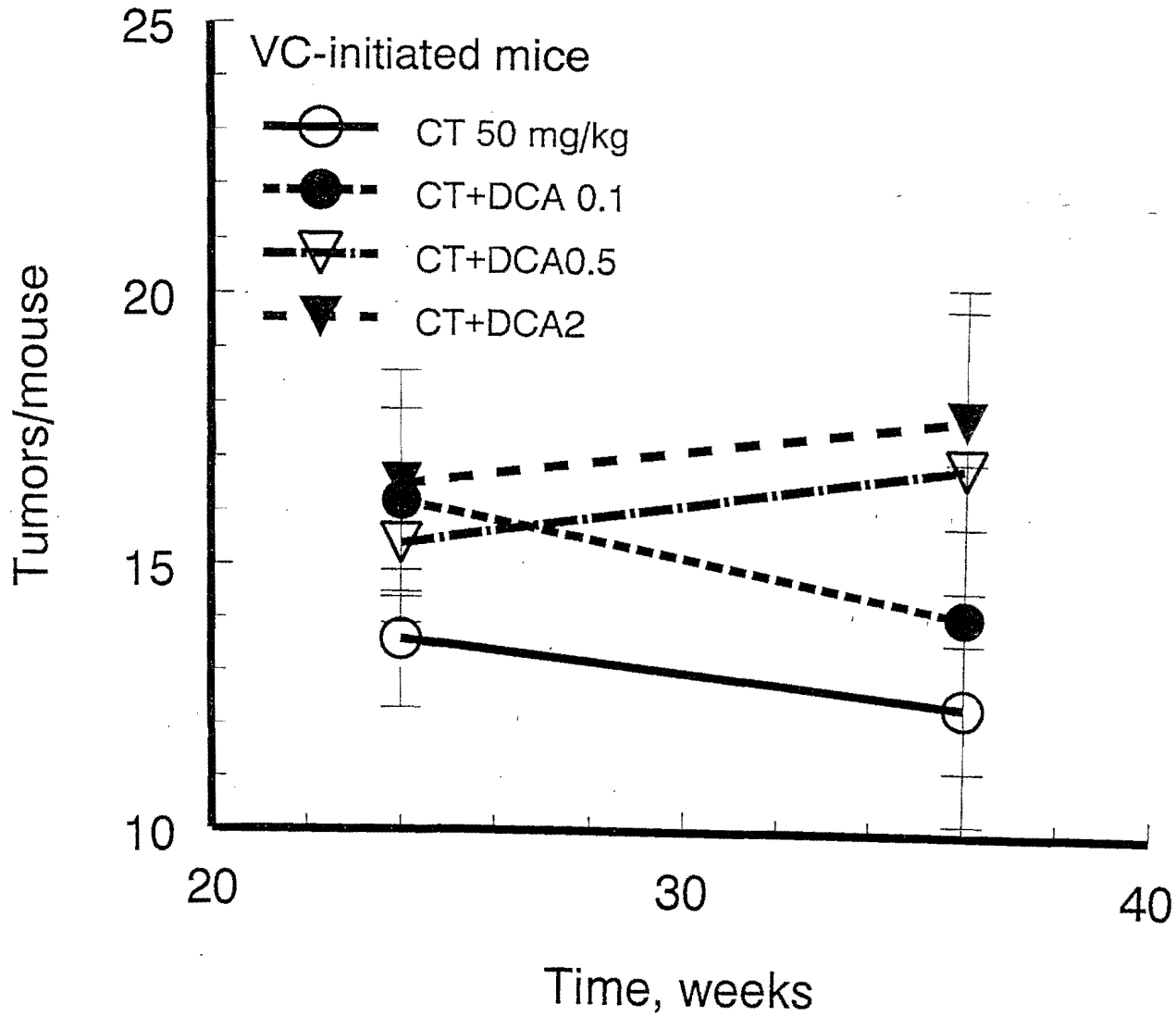

Figure 11. Tumors/Mouse in Mice Treated with a Single Concentration of CT and Varying Concentrations of DCA. Tumor response in the liver of male B6C3F1 mice initiated with 3 mg/kg vinyl carbamate at 15 days of age followed by treatment with a single 50 mg/kg dose of CT administered in a 5% Alkamuls® in water vehicle with concentrations of DCA in their drinking water varying from 0-2 g/L. (Mean ± SEM). Symbols indicate that the response was significant ($P<0.05$) related to treatment (*), whether the effect was modified by week (†), or whether there were interactions between treatment and time (‡). See Table 2 for actual P values.

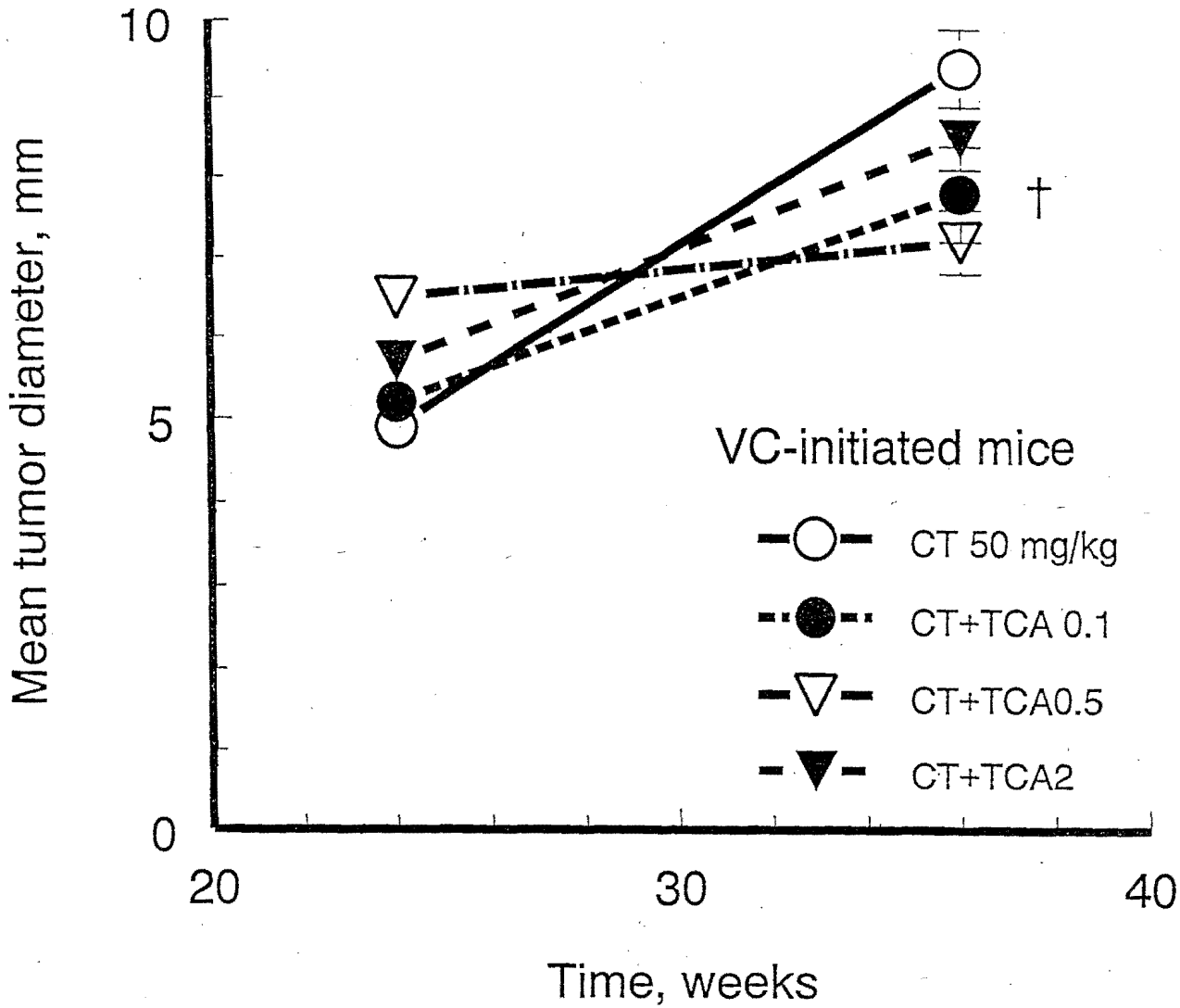

Figure 12. Tumor size in Mice Treated with a Single Concentration of CT and Varying Concentrations of DCA. Tumor response in the liver of male B6C3F1 mice initiated with 3 mg/kg vinyl carbamate at 15 days of age followed by treatment with a single 50 mg/kg dose of CT administered in a 5% Alkamuls® in water vehicle with concentrations of DCA in their drinking water varying from 0-2 g/L. (Mean ± SEM). Symbols indicate that the response was significant (P<0.05) related to treatment (*), whether the effect was modified by week (†), or whether there were interactions between treatment and time (‡). See Table 2 for actual P values

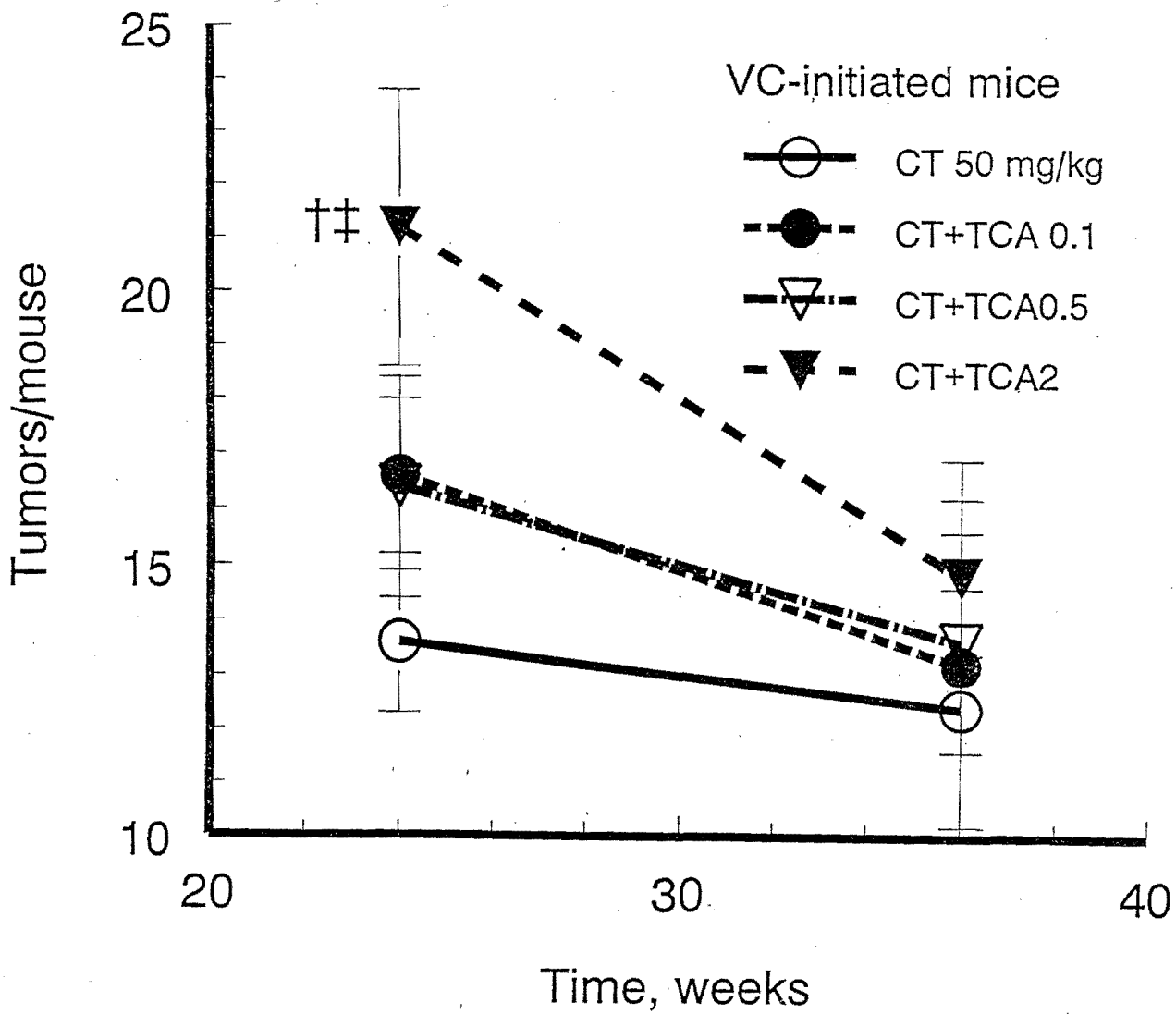

Figure 13. Tumors/Mouse in Mice Treated with a Single Concentration of CT and Varying Concentrations of TCA. Tumor response in the liver of male B6C3F1 mice initiated with 3 mg/kg vinyl carbamate at 15 days of age followed by treatment with a single 50 mg/kg dose of CT administered in a 5% Alkamuls in water vehicle with varying concentrations of TCA ranging from 0 to 2 g/L (Mean ± SEM). Symbols indicate that the response was significant ($P<0.05$) related to treatment (*), whether the effect was modified by week (†), or whether there were interactions between treatment and time (‡). See Table 2 for actual P values.

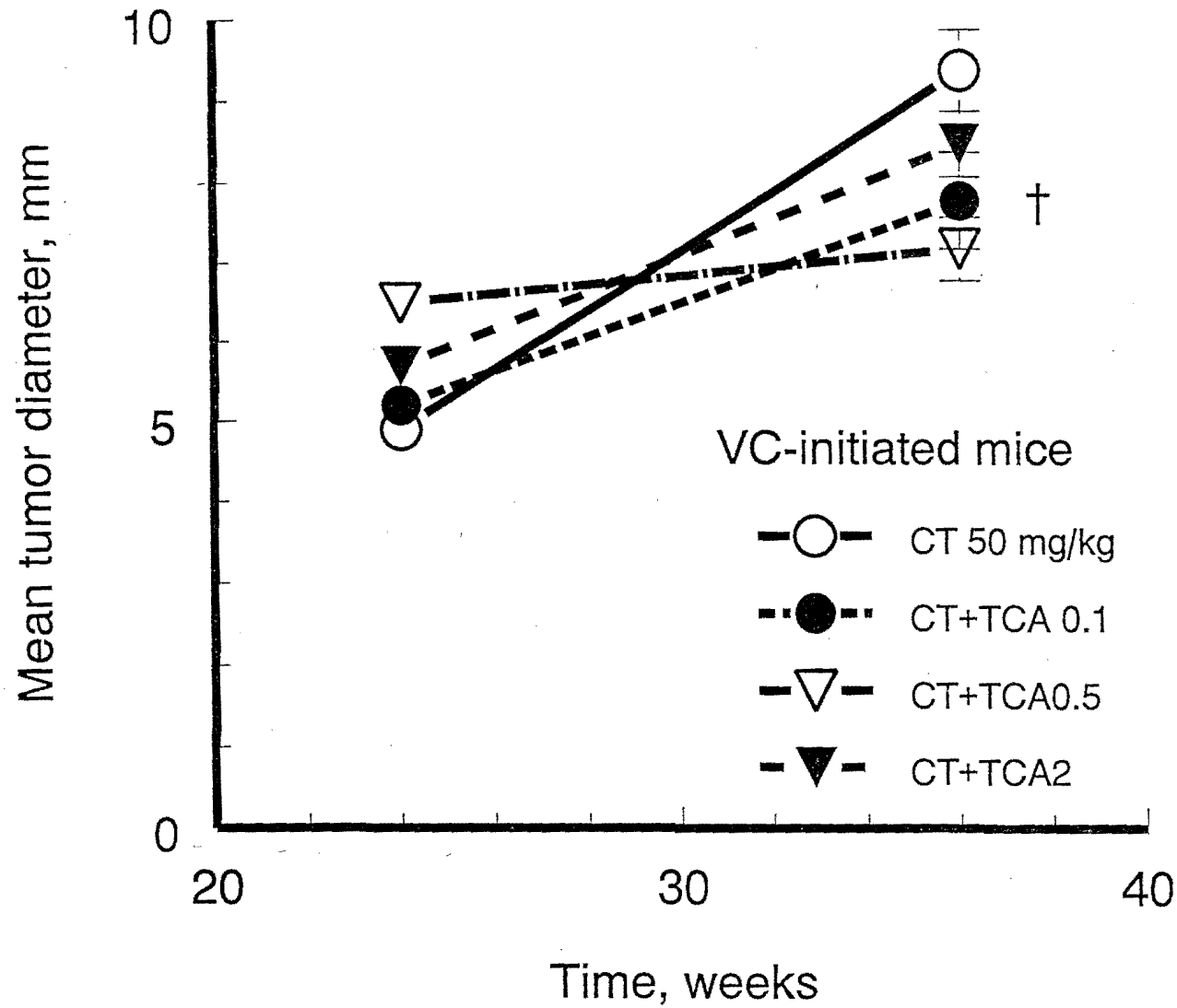

Figure 14. Tumor Size in Mice Treated with a Single Concentration of CT and Varying Concentrations of TCA. Tumor response in the liver of male B6C3F1 mice initiated with 3 mg/kg vinyl carbamate at 15 days of age followed by treatment with a single 50 mg/kg dose of CT administered in a 5% Alkamuls in water vehicle with varying concentrations of TCA ranging from 0 to 2 g/L (Mean ± SEM). Symbols indicate that the response was significant ($P<0.05$) related to treatment (*), whether the effect was modified by week (†), or whether there were interactions between treatment and time (‡). See Table 2 for actual P values